Introduction of a Tryptophan Reporter Group into Loop 1 of the recA Protein

EXAMINATION OF THE CONFORMATIONAL STATES OF THE recA-ssDNA COMPLEX BY FLUORESCENCE SPECTROSCOPY*

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Site-directed mutagenesis was used to replace His-163 in the Loop 1 region of the recA protein with a tryptophan residue. The [H163W]recA protein binds single-stranded DNA (ssDNA), catalyzes ssDNA-dependent ATP hydrolysis, and is fully active in the three-strand exchange reaction. In addition, the fluorescence properties of the Trp-163 reporter group are very sensitive to the binding of nucleotide cofactors to the [H163W]recA-ssDNA complex. The fluorescence of Trp-163 is modestly quenched by the binding of ADP (21%) and strongly quenched by the nonhydrolyzable ATP analog, ATPγS (70%); since ADP and ATPγS stabilize the closed and open conformations of the recA-ssDNA complex, respectively, the quenched states observed with these nucleotides likely reflect differences in the fluorescence properties of tryptophan in these two states. ATP has a more complex time-dependent effect on Trp-163 fluorescence. When ATP is added to [H163W]recA-ssDNA complexes, there is an immediate quenching of Trp-163 fluorescence (44%) which is intermediate in intensity between that observed with ADP and ATPγS. The ATP-induced quenching gradually decreases with time as the pool of ATP is converted to ADP by the ATP hydrolysis activity of the [H163W]recA protein. These results are discussed with regard to the nucleotide cofactor-dependent conformational transitions of the recA-ssDNA complex.

The recA protein of Escherichia coli is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The recA protein is composed of 352 amino acids and has a molecular mass of 37,842 daltons. The purified recA protein binds cooperatively to ssDNA, forming a polymeric, filament-like structure that catalyzes the hydrolysis of ATP to ADP and Pᵢ. In addition, the recA protein will promote a variety of DNA pairing reactions that presumably reflect in vivo recombination functions (Griffith and Harris, 1988; Roca and Cox, 1990; Kowalczykowski, 1991).

The most extensively investigated DNA pairing activity of the recA protein is the three-strand exchange reaction, in which a circular ssDNA molecule and a homologous linear dsDNA molecule are recombined to yield a nicked circular dsDNA molecule and a linear ssDNA molecule. This reaction is dependent on ATP and is strongly stimulated by the E. coli SSB protein. Although the molecular mechanism of the three-strand exchange reaction is not well understood, the reaction is known to proceed in three phases. In the first phase, the circular ssDNA substrate is coated with recA protein to form a presynaptic complex; SSB aids in the formation of this complex by melting out regions of secondary structure in the ssDNA that otherwise impede the binding of recA protein. In the second phase, the presynaptic complex interacts with a dsDNA molecule, the homologous sequences are brought into register, and pairing between the circular ssDNA and the complementary strand from the dsDNA is initiated. In the third phase, the complementary linear strand is completely transferred to the circular ssDNA by unidirectional branch migration to yield the nicked circular dsDNA and displaced linear ssDNA products (Griffith and Harris, 1988; Roca and Cox, 1990; Kowalczykowski, 1991).

The presynaptic complex formed between recA protein and ssDNA is the active recombinational entity in the strand exchange reaction. The recA protein binds cooperatively to ssDNA, forming a right-handed helical protein filament with one recA monomer per four nucleotides of ssDNA and six recA monomers per turn of the filament. In the absence of nucleotide cofactor or in the presence of ADP, the helical filament adopts a "collapsed" or "closed" conformation (helical pitch 65 Å) that is inactive in strand exchange. In the presence of ATP or the nonhydrolyzable ATP analog, ATPγS, however, the filament assumes an "extended" or "open" conformation (helical pitch 95 Å) that is active in strand exchange (Egelman, 1993).

Two of our recently described mutant recA proteins, [G160N]recA protein and [H163A]recA protein, have been useful for examining the role of ATP-dependent conformational changes in the three-strand exchange reaction. Both of these mutant proteins bind to ssDNA and catalyze ssDNA-dependent ATP hydrolysis with turnover numbers that are similar to that for the wild-type protein. Neither mutant protein, however, is able to isomerize in the presence of ATP to the open conformation under standard reaction conditions (pH 7.5, 37 °C), and, consequently, neither mutant protein is active in the ATP-dependent three-strand exchange reaction under these conditions. Interestingly, the mutant proteins are able to carry out ATP-dependent strand exchange at pH 6.0 to 6.8, with the induction of strand exchange activity correlating directly with the activation of the ATP-dependent isomerization of the mutant proteins to the strand exchange active conformational state at the lower pH. This activation correlates with a pH-dependent change in the S0.5 value for ATP, with the mutant proteins becoming active in isomerization and in strand exchange at pH values where the S0.5(ATP) decreases below 100
μm (Bryant, 1988; Muench and Bryant, 1990, 1991; Pinsince et al., 1993; Meah and Bryant, 1993). We have demonstrated a similar dependence of the strand exchange reaction on the S_{H163} value of the cofactor in our studies of the wild-type recA protein with alternate nucleoside triphosphates. The nucleoside triphosphates ATP, PTP, ITP, and GTP are each hydrolyzed by the recA protein with the same turnover number (20 min^{-1}). ATP and PTP, which have S_{H163} values below 100 μm, support isomerization of the recA-ssDNA complex to the active conformational state and function as cofactors for the strand exchange reaction. ITP and GTP, in contrast, have S_{H163} values above 100 μm, are ineffective in isomerization, and do not function as cofactors for strand exchange (Menge and Bryant, 1992). Taken together, these studies have allowed us to identify the isomerization of the recA-ssDNA complex to the open conformational state as an obligatory step on the strand exchange reaction pathway. Furthermore, they indicate that nucleoside triphosphates with S_{H163} values greater than 100 μm may be intrinsically unable to stabilize the recA-ssDNA complex in the open conformational state and will therefore be nonfunctional as cofactors for the strand exchange reaction.

The x-ray structure of the recA protein shows that Gly-160 and His-163 are both part of a disordered loop region (Loop 1) which extends from amino acid 157 to 164 (Story et al., 1992). Since the [G160N]recA and [H163A]recA proteins are functional in strand exchange at low pH, it is clear that neither Gly-160 nor His-163 is absolutely required for recA protein function. Instead, these two mutations may simply perturb the structure of the recA protein enough to raise the S_{H163}(ATP) above the threshold value of 100 μm at pH 7.5 (Meah and Bryant, 1993). In this paper, we report a new mutant recA protein in which His-163 has been replaced by a tryptophan residue. We show that the [H163W]recA protein has a S_{H163}(ATP) value lower than 100 μm at pH 7.5 and, accordingly, is fully functional in the strand exchange reaction under standard reaction conditions. Furthermore, Trp-163 serves as a reporter group in Loop 1 of the recA protein which allows us to follow the conformational transitions of the recA-ssDNA complex by fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Materials—Wild-type recA protein was prepared as described previously (Bryant, 1988). ATP, ADP, N-acetyl tryptophanamide, and carbencillin were from Sigma. 3-[H]ATP was from ICN, and E. coli SSB was from Pharmacia LKB Biotechnology Inc. Endonuclease BamHI and T4 DNA ligase were from Bethesda Research Laboratories. E. coli strain BNN124 (Elledge and Davis, 1987) was obtained from Dr. Steve Elledge (Stanford University). Unlabeled and 3H-labeled circular 

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\text{TABLE I}
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<tr>
<th>Complex</th>
<th>ΔF_{H163}</th>
<th>ΔF_{W163}</th>
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<tr>
<td>Wild-type recA</td>
<td>+ADP</td>
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</tr>
<tr>
<td></td>
<td>+ATP</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+ATP+S</td>
<td>9</td>
</tr>
<tr>
<td>[H163W]recA</td>
<td>+ADP</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+ATP</td>
<td>20</td>
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<tr>
<td></td>
<td>+ATP+S</td>
<td>29</td>
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\text{ΔF}_{H163} = 2.68F_{H163}(H163W) - 1.68F_{W163}(H163W) - \Delta F_{W163}
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Fluorescence Analysis—Fluorescence studies were conducted on a Perkin-Elmer LS 50 luminescence spectrometer equipped with a variable temperature holder. The concentrations of stock solutions of the purified wild-type and [H163W]recA proteins were determined by absorbance using the extinction coefficients 0.59 and 1.2 A_{290} mg^{-1} ml^{-1}, respectively. Each fluorescence emission spectrum was corrected for background fluorescence by subtracting the corresponding buffer spectrum. Inner filter corrections at high concentrations of DNA or nucleotide were made using the relationship \( F_{corr} = F_{obs} \text{anti-log}(A_{290} + A_{abs}/2) \) (valid for absorbances \( \leq 0.1 \) (Lakowicz, 1983) and were less than 3% in all cases.

RESULTS

Design and Preparation of [H163W]recA Protein—We wished to introduce into the recA protein a tryptophan reporter group that would allow us to monitor the conformational transitions of the recA-ssDNA complex by fluorescence spectroscopy. The wild-type recA protein does contain 2 tryptophan residues, Trp-290 and Trp-308, at all stages of purification. The concentration of the purified [H163W]recA protein was determined by amino acid analysis using the Pico-Tag system (Biddinger et al., 1984) and used to calculate the extinction coefficient at 280 nm (ε = 1.2 A_{290} mg^{-1} ml^{-1}).

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\text{Fluorescence Analysis—Fluorescence studies were conducted on a Perkin-Elmer LS 50 luminescence spectrometer equipped with a variable temperature holder. The concentrations of stock solutions of the purified wild-type and [H163W]recA proteins were determined by absorbance using the extinction coefficients 0.59 and 1.2 A_{290} mg^{-1} ml^{-1}, respectively. Each fluorescence emission spectrum was corrected for background fluorescence by subtracting the corresponding buffer spectrum. Inner filter corrections at high concentrations of DNA or nucleotide were made using the relationship } \]
of the helical filament and is likely to be in contact with the ssDNA in the recA-ssDNA complex (Story et al., 1992). In addition, our studies of the [H163A]recA and [G160N]recA proteins suggested that this region might be sensitive to the conformational changes which occur in response to the binding of various nucleotide cofactors to the recA-ssDNA complex (Bryant, 1988; Muech and Bryant, 1990, 1991; Pinsince et al., 1993; Meah and Bryant, 1993). Based upon these considerations, His-163 was selected for replacement by a tryptophan reporter group. The purified [H163W]recA protein is shown in Fig. 1.

Functionality of the [H163W]recA Protein—The ssDNA binding, ssDNA-dependent ATP hydrolysis, and ATP-dependent three-strand exchange activities of the purified [H163W]recA protein were evaluated under standard reaction conditions (25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 30 μM 3H-labeled dX ssDNA, and the indicated concentrations of wild-type recA or [H163W]recA protein). After a 10-min incubation at 25 °C, the reaction mixtures were filtered on ROH-treated nitrocellulose filters (HAWP 0.45 μm, Millipore), washed with 2 ml of reaction buffer, dried, and assayed for radioactivity by liquid scintillation counting. The points represent the percentage of the total ssDNA that was retained on the filters.

Fig. 2. Single-stranded DNA binding by the wild-type and [H163W]recA proteins. Nitrocellulose filter binding assays were carried out as described previously (Bryant and Lehman, 1986). The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 10 mM MgCl₂, 30 μM 3H-labeled dX ssDNA, and the indicated concentrations of wild-type recA or [H163W]recA protein. After a 10-min incubation at 25 °C, the reaction mixtures were filtered on ROH-treated nitrocellulose filters (HAWP 0.45 μm, Millipore), washed with 2 ml of reaction buffer, dried, and assayed for radioactivity by liquid scintillation counting. The points represent the percentage of the total ssDNA that was retained on the filters.

The ssDNA-dependent ATP hydrolysis activity of the [H163W]recA protein was measured as a function of ATP concentration. As shown in Fig. 3, the [H163W]recA protein catalyzed ssDNA-dependent ATP hydrolysis with a turnover number of 20 min⁻¹, a value indistinguishable from that for the wild-type recA protein (Menge and Bryant, 1988). The S°₅(ATP) for the [H163W]recA protein was 70 μM, compared to a value of 45 μM for the wild-type recA protein. Thus, the [H163W]recA mutation has only a minor effect on the ssDNA-dependent ATP hydrolysis activity of the recA protein.

The [H163W]recA protein was examined for ATP-dependent three-strand exchange activity under standard reaction conditions (pH 7.5, 37 °C). In the three-strand exchange assay, a circular dX ssDNA molecule and a linear dX dsDNA molecule are recombined to form a nicked circular dsDNA molecule and a linear ssDNA molecule; the substrates and products of this reaction are readily monitored by agarose gel electrophoresis (Cox and Lehman, 1981). As shown in Fig. 4, the [H163W]recA protein exhibited strand exchange activity, with substantial strand exchange occurring within 30 min; this activity is comparable to that exhibited by the wild-type protein (Menge and Bryant, 1992). Consistent with the three-strand exchange activity, we have shown that the [H163W]recA protein is able to complement a recA deletion in vivo, as judged by UV sensitivity assays (data not shown). Thus, the [H163W]recA protein appears to be fully functional in recA protein-dependent DNA strand exchange activities.

Fluorescence Properties of the Wild-type and [H163W]recA Proteins—The fluorescence emission spectra of the wild-type and [H163W]recA protein at pH 7.5 and 37 °C are shown in Fig. 5. The spectra were obtained at an excitation wavelength of 295 nm, so that only tryptophan fluorescence would be measured (Lakowicz, 1984). The spectrum of the wild-type recA protein has an emission maximum at 344 ± 1 nm. In the [H163W]recA protein spectrum, the emission maximum is shifted to 347 ± 1 nm. The fluorescence intensity of the [H163W]recA protein at its emission maximum is 68% greater than that of the wild-type
RecA protein, reflecting the additional tryptophan at position 163. The difference spectrum obtained by subtracting the spectrum of the wild-type protein from that of the [H163W]RecA protein indicates that Trp-163 has an emission maximum of 349 nm. This value is similar to the emission maximum of 350 nm that was obtained for N-acetyl tryptophanamide in the same reaction buffer (spectrum not shown) and suggests that Trp-163 is in a solvent-accessible region of the protein (Lakowicz, 1984).

Fluorescence Properties of the Wild-type and [H163W]RecA-ssDNA Complexes—The wild-type recA and [H163W]RecA proteins (1 μM) were added to dX ssDNA (10 μM) in order to form the corresponding recA-ssDNA complexes. Various nucleotides were then added to the complexes, and the effects on the total tryptophan fluorescence of the proteins (excitation wavelength: 295 nm) were measured.2

As shown in Table I, the addition of 1 mM ADP, ATP, or ATPγS to the wild-type recA-ssDNA complex resulted in only minor changes in tryptophan fluorescence. This indicates that the fluorescence properties of the intrinsic tryptophan residues of the wild-type recA protein (Trp-290 and Trp-308) are relatively insensitive to nucleotide cofactor-dependent changes in the conformation of the recA-ssDNA filament.

In contrast to the results obtained with the wild-type recA protein, there was a 9% decrease in total tryptophan fluorescence intensity when ADP (1 mM) was added to the [H163W]RecA-ssDNA complex, a 20% decrease when ATP (1 mM) was added, and a 29% decrease when the nonhydrolyzable ATP analog, ATPγS (1 mM), was added (Table I). The emission maximum wavelength of the [H163W]RecA protein remained unchanged after the addition of these nucleotides. Assuming that the fluorescence properties of Trp-290 and Trp-308 are not altered in the [H163W]RecA protein, these results correspond to a 21%, 44%, and 70% quenching of Trp-163 fluorescence in the presence of ADP, ATP, and ATPγS, respectively. Identical results were obtained with higher nucleotide concentrations, indicating that these quench values are representative of [H163W]RecA-ssDNA complexes that are saturated with each nucleotide. There was no effect of Pi (1 mM) on any of the quenched states (data not shown).

The fluorescence of the [H163W]RecA protein (1 μM) was also measured in the presence of a fixed concentration of ATP (1 mM) and various concentrations of dX ssDNA. As shown in Fig. 6, the quenching of Trp-163 fluorescence increased with increasing ssDNA concentrations, reaching a maximum at 10 μM ssDNA. This curve closely resembles the saturation curve that was obtained by measuring the steady state rate of ssDNA-dependent ATP hydrolysis (1 mM ATP) by the [H163W]RecA protein (1 μM) as a function of ssDNA concentration (Fig. 6). These results indicate that 10 μM dX ssDNA is the minimum concentration that is able to fully complex 1 μM [H163W]RecA protein. Although the binding site size for the recA protein is approximately 4 nucleotides of ssDNA per recA monomer (Bryant and Lehman, 1986), dX ssDNA apparently contains regions of secondary structure that render approximately 65% of the ssDNA molecule unavailable for recA protein binding under our reaction conditions (Menge and Bryant, 1988). Thus, a dX ssDNA concentration of 10 μM will provide approximately 4 μM ssDNA for recA binding, an amount sufficient to saturate 1 μM recA protein. These concentrations of [H163W]RecA protein and ssDNA were used in the fluorescence experiments described below.

ATP-mediated Quenching of Trp-163 Fluorescence—As shown in Fig. 7, the quenching of Trp-163 fluorescence which occurred when ATP (1 mM) was added to the [H163W]RecA-ssDNA complex exhibited a time-dependent behavior. Following the initial 44% decrease in fluorescence, which occurred within the mixing time of the experiment, there was a further increase in quenching to 48% over the next 10 min. The ATP hydrolysis reaction catalyzed by the [H163W]RecA protein proceeded at a constant rate (18 min−1) during this phase of fluorescence, indicating that this slow secondary phase of quenching was not related to any changes in the catalytic properties of 2The addition of either ssDNA (10 μM) or ATP (1 mM) alone to the wild-type recA protein (1 μM) resulted in an apparent 15–20% decrease in total tryptophan fluorescence at 344 nm. Similar results were obtained for the [H163A]RecA protein. These effects, which likely reflect gross changes in the aggregation state of the recA protein (Brenner et al., 1988), will not be considered in this paper.
FIG. 5. Fluorescence emission spectra of the wild-type and [H163W]recA proteins. The samples contained 40 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, 10 mM MgCl₂, 1 μM wild-type recA protein or [H163W]recA protein. The excitation wavelength was 295 nm, and the excitation and emission bandwidths were each set at 5 nm. Each fluorescence spectrum was corrected for background fluorescence and the Raman scattering peak by subtracting the corresponding buffer spectrum.

FIG. 6. Dependence of ATP-mediated quenching of tryptophan 163 fluorescence on ssDNA concentration. The reaction solution contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, 10 mM MgCl₂, 1 μM [H163W]recA protein, 1 mM ATP, and the indicated concentrations of 4X ssDNA. The reactions were carried out at 37 °C. ATP hydrolysis and the quenching of Trp-163 fluorescence were measured as described in Figs. 3 and 5, respectively. The percent quenching of Trp-163 fluorescence at each ssDNA concentration is represented by closed circles, and the open squares represent the rate of ATP hydrolysis measured at each ssDNA concentration.

The transient increase in Trp-163 fluorescence may be related to the stimulation of the strand exchange reaction that is observed in the presence of low concentrations of ADP (Cox et al., 1983).

FIG. 7. Dependence of ATP-mediated quenching of Trp-163 fluorescence on time. The reaction solution contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, 10 mM MgCl₂, 10 μM [H163W]recA protein, 1 mM ATP. The reaction was carried out at 37 °C. Fluorescence emission was measured at 346 nm using an excitation wavelength of 295 nm. The percent quenching of Trp-163 fluorescence is defined by \( I/I_0 \), where \( I_0 \) is the fluorescence intensity measured before addition of ATP, and \( I \) is the fluorescence intensity measured after addition of ATP. The closed circles represent the percent quenching of Trp-163 fluorescence, and the open squares represent the amount of ATP hydrolyzed as a function of time.

The transient increase in Trp-163 fluorescence quenching may be related to the stimulation of the strand exchange reaction that is observed in the presence of low concentrations of ADP (Cox et al., 1983).
Fluorescence on ATP concentration. The reactions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 10 mM MgCl₂, 30 μM ssDNA, 1 μM [H163W]recA protein, and the indicated concentrations of ATP. The reactions were carried out at 37 °C. Panel A, time courses. The percent quenching of Trp-163 fluorescence is plotted as a function of reaction time for reactions containing 50 (○), 100 (□), 250 (△), 500 (●), 1000 (○), and 2000 (□) μM ATP. The quenching of Trp-163 fluorescence was measured as described in Fig. 7. Panel B, titration curve. Each of the time courses in panel A was extrapolated to zero time in order to estimate the initial quenching of Trp-163 fluorescence at each ATP concentration. The initial quench values obtained at each ATP concentration are plotted as closed circles. The rates of ATP hydrolysis at each ATP concentration are represented by open squares.

Trp-163 fluorescence are related to the changes in the relative concentrations of ATP and ADP in the reaction solution which occur as a result of the ssDNA-dependent ATP hydrolysis activity of the [H163W]recA protein.

Dependence of Tryptophan 163 Fluorescence on ATP Concentration—The fluorescence of the [H163W]recA protein (1 μM) was next measured in the presence of a fixed concentration of 3X ssDNA (10 μM) and various concentrations of ATP. Time courses showing the initial quenching of Trp-163 fluorescence as well as the slower secondary phase of fluorescence quenching at each ATP concentration are presented in Fig. 8A. Each of the secondary fluorescence phases was extrapolated to zero time in order to estimate the initial quenching of Trp-163 fluorescence at each ATP concentration.

As shown in Fig. 8B, the initial quenching of Trp-163 fluorescence increased with increasing ATP concentration, reaching a maximum of 44% at ATP concentrations greater than 1 mM. The half-maximal quenching occurred at an ATP concentration of 90 μM. This fluorescence titration curve closely resembles the saturation curve that was obtained by measuring the steady state rate of ssDNA-dependent ATP hydrolysis by the [H163W]recA protein as a function of ATP concentration (Fig. 8B). These results indicate that the initial quenching of Trp-163 fluorescence by ATP is directly related to the saturation of the nucleotide binding sites in the polymeric [H163W]recA-ssDNA complex.

DISCUSSION

We have used site-directed mutagenesis to replace His-163 of the recA protein with a tryptophan reporter group. The [H163W]recA protein binds ssDNA, catalyzes ssDNA-dependent ATP hydrolysis, and is fully active in the three-strand exchange reaction under standard reaction conditions (3X DNA, pH 7.5). Furthermore, the binding of various nucleotide cofactors to the [H163W]recA-ssDNA complex results in the quenching of the fluorescence of Trp-163. The extent of quenching is lowest with ADP (21%), intermediate with ATP (44%), and highest with the nonhydrolyzable ATP analog, ATPγS (70%). There is no overlap between the fluorescence emission spectrum of the [H163W]recA protein and the absorption spectra of either the adenine nucleotides or the ssDNA (data not shown). Thus, the quenching of Trp-163 fluorescence is likely due to a conformational change of the protein which alters the local environment of Trp-163, rather than to energy transfer from Trp-163 to the nucleotide cofactor or ssDNA (Lakowicz, 1984).

Since ADP and ATPγS stabilize the closed and open conformations of the recA-ssDNA complex, respectively, the differential quenching observed with these nucleotides likely reflects differences in the fluorescence properties of Trp-163 in these two states. Furthermore, the quenching observed with ADP indicates that the conformation of the closed recA-ssDNA-ADP complex may not be structurally equivalent to the closed recA-ssDNA complex that exists in the absence of nucleotide cofactor (Lee and Cox, 1990a, 1990b).

The quenching of Trp-163 fluorescence by ATP is more complex than that observed with either ADP or ATPγS. Unlike ADP and ATPγS, ATP is a substrate for the hydrolysis activity of the recA protein, and the time-dependent changes in ATP-mediated quenching of Trp-163 fluorescence reflect the changing concentrations of ATP and ADP in the reaction solution as the ATP hydrolysis reaction progresses. The maximal level of Trp-163 fluorescence quenching observed with ATP before a significant concentration of ADP has accumulated in solution, is still less than that observed with ATPγS. Under these conditions, however, some of the active sites in the polymeric recA-ssDNA complex will contain newly generated ADP molecules, whereas others will contain as yet unhydrolyzed ATP molecules. Thus, the intermediate level of quenching obtained with ATP may represent a mixture of fluorescence signals from a highly quenched [H163W]recA-ssDNA-ATP state and a lesser quenched [H163W]recA-ssDNA-ADP state, which coexist during steady state ATP hydrolysis.

The ATP-mediated quenching of Trp-163 fluorescence exhibits a dependence on ATP concentration that closely resembles that for the [H163W]recA protein-catalyzed ssDNA-dependent ATP hydrolysis reaction. This indicates that the quenching is linearly related to the saturation of nucleotide binding sites in the polymeric [H163W]recA-ssDNA complex. Thus, sub saturating amounts of ATP do not appear to induce a cooperative quenching in multiple recA monomers. Instead, the fluorescence of Trp-163 in a particular recA monomer within the [H163W]recA-ssDNA complex may depend on the nucleotide bound to that monomer and not on the overall structure of the filament.

The ssDNA-dependent ATP hydrolysis reaction catalyzed by the [H163W]recA protein terminates when approximately 65% of the ATP originally present has been converted to ADP. This phenomenon has been reported for the wild-type recA protein...
and has been attributed to a dissociation of the recA-ssDNA complex which occurs at high ADP concentrations as a result of a structural incompatibility of the ATP- and ADP-induced conformational states. This explanation, however, was based largely on studies of the interaction of the recA protein with dsDNA (Lee and Cox, 1990a, 1990b). Our studies show that the level of Trp-163 fluorescence quenching after termination of [H163W]recA protein-catalyzed ssDNA-dependent ATP hydrolysis (65% hydrolysis) is substantially greater than that observed when an equivalent concentration of ATP and ADP are added to the [H163W]recA protein in the absence of ssDNA (data not shown). This result implies that the [H163W]recA protein remains bound to the ssDNA even after the ATP hydrolysis reaction has terminated. Consistent with this idea, we have shown that there is full retention of ssDNA by the recA protein in the nitrocellulose filter binding assay at ATP-ADP ratios that do not support the ssDNA-dependent ATP hydrolysis reaction (data not shown). Since the recA protein can bind to ssDNA in either an active (open) or inactive (closed) conformation, it may not be necessary to invoke dissociation of the recA-ssDNA complex in order to explain the inhibition of ATP hydrolysis which occurs when the ATP-ADP ratio reaches 35:65. Instead, the recA-ssDNA complex may remain intact, but in an inactive state, possibly because a high percentage occupancy of active sites with ADP may inactivate the ATP hydrolysis activity of the complex. In contrast, the binding of recA protein to dsDNA is coupled to DNA unwinding and requires the recA protein to be in the ATP-dependent open conformational state. In this case, the recA protein may indeed dissociate from dsDNA if the complex reverts to an inactive conformational state when the ATP:ADP ratio reaches 35:65 (Lee and Cox, 1990a, 1990b).

In contrast to the [H163W]recA protein, our previously described [H163A]recA protein is inactive in strand exchange under standard reaction conditions (Bryant, 1988). The $S_{0.5}(\text{ATP})$ value for the [H163W]recA protein (70 μM), however, is lower than the $S_{0.5}(\text{ATP})$ for the [H163A]recA protein (150 μM) at pH 7.5. This difference is significant in that our previous studies indicated that a nucleoside triphosphate must have an $S_{0.5}$ value of 100 μM or less in order to allosterically stabilize the recA-ssDNA complex in the open conformation and function as a cofactor for the strand exchange reaction (Menge and Bryant, 1992). Thus, the results in this paper confirm our earlier conclusion that His-163 is not essential to recA function and support the idea that the effect of a particular His-163 mutation on strand exchange activity depends on the extent to which the mutation perturbs the $S_{0.5}$ for ATP (Meah and Bryant, 1993).

We are now examining the fluorescence properties of the [H163W]recA protein in the presence of a series of alternate nucleoside triphosphate cofactors having a range of $S_{0.5}$ values above and below 100 μM. These studies will enable us to more precisely relate the quenching of Trp-163 fluorescence to the isomerization of the [H163W]recA-ssDNA complex.

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REFERENCES


