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Direct Observation of Complexes of ssb and recA Proteins with a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative†

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ABSTRACT: Evidence is presented from fluorescence and kinetic experiments that ssb binds to a recA–ssDNA–ATP complex causing a major structural change in which some 40% of the bound recA is released. On addition of ssb to recA–ϵDNA–ATP (containing the fluorescent analogue of ssDNA ϵDNA), there is a slow first-order decrease in fluorescence ($t_{1/2} \sim 3$ min). This is accompanied by a loss in the ATPase activity of recA protein. The resultant complex does not exchange ϵDNA for added ssDNA. Measurement of the DNA-stimulated ATPase activity on addition of excess ssDNA reveals that 40% of the previously bound recA has been released. The stoichiometry of recA bound to ϵDNA thus changes from 1 mol per six nucleotides to 1 per 10 on addition of ssb. Formation of the ssb–recA–ϵDNA complex is dependent on ATP, and the rate varies with the concentration of ssb.

General recombination and repair of DNA damage in *Escherichia coli* require the presence of the recA gene product (Clark, 1973; Radding, 1978; Radman, 1975; Witkin, 1976). This is a protein of M_r 37 800 (Horii et al., 1980; Sancar et al., 1980) that has a large number of activities in vitro. It is a ssDNA[1] and dsDNA-dependent ATPase (Ogawa et al., 1979; Roberts et al., 1979; Weinstock et al., 1979; McEntee et al., 1979; Shibata et al., 1979a,b; West et al., 1980); it catalyzes D loop formation and strand exchange (Cassuto et al., 1981; Cox & Lehman, 1981; DasGupta et al., 1981; West et al., 1981) and has also been shown to possess an ssDNA- and ATP-dependent protease activity for certain repressor proteins (Roberts et al., 1979; Craig & Roberts, 1980).

Recent studies have indicated that interactions with other proteins affect the activity of recA. The interaction with the ssDNA binding protein (ssb) from *E. coli* has been shown to be important both in vitro and in vivo. A combination of recA and ssb will catalyze D loop formation, strand exchange, and the protease reaction far more efficiently than recA protein alone, although the ATPase reaction is inhibited (Shibata et al., 1980; McEntee et al., 1980; Resnick & Sussman, 1982; Cox & Lehman, 1982). Cox & Lehman (1982) have suggested that this may be related to a tightening up of the recA–ssDNA complex, but, as yet, attempts to isolate a

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); NTP, nucleotide triphosphate; ϵDNA, product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; ssb, ssDNA binding protein; Tris, tris(hydroxymethyl)aminomethane.

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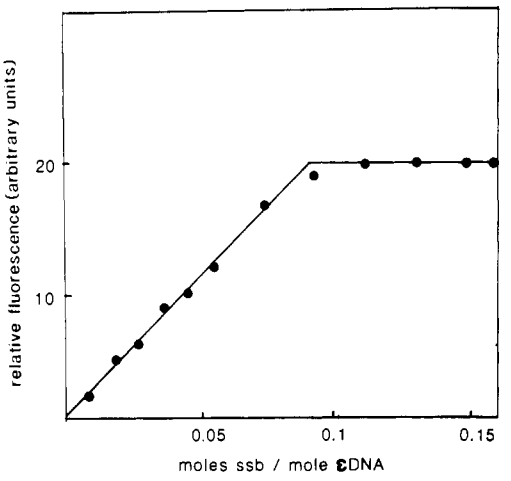

FIGURE 1: Titration of εDNA with ssb (3 μM).

recA–ssb complex with protein cross-linking reagents have not been successful.

In this paper, we use εDNA (a fluorescent derivative of ssDNA) to show an interaction of ssb with the recA–εDNA–ATP complex. We also present a preliminary characterization of the fluorescent complex produced.

Materials and Methods

εDNA was prepared as described previously (Silver & Fersht, 1982). recA protein was prepared as described previously (Cotterill et al., 1982). ssb was a kind gift from Dr. F. Grosse. ATP and ATPγS were obtained from Boehringer, Mannheim. Poly(dT) was from P-L Biochemicals Ltd. Single-stranded calf thymus DNA was prepared from highly polymerized calf thymus dsDNA as described by Cotterill et al. (1982).

All concentrations of nucleotides and polynucleotides were determined spectroscopically by using published extinction coefficients. [γ-^{32}P]ATP was obtained from Amersham Int. (U.K.). The concentration of ssb was calculated with an extinction coefficient of 0.98 mg^{-1} mL^{-1} cm^{-1} (Krauss et al., 1981) and that of recA with one of 0.517 mg^{-1} mL^{-1} cm^{-1} (Kuramitsu et al., 1981; Cotterill et al., 1982).

The stock buffer was 10 mM Tris-HCl (pH 8.1), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Where specified, 10 mM Ca^{2+} or 10 mM Mn^{2+} was substituted for $MgCl_2$, and 10 mM sodium maleate (pH 6.2) was substituted for Tris-HCl.

Fluorescence Measurements. Fluorescence studies were performed as described previously (Silver & Fersht, 1982). The excitation wavelength was 310 nm, and that of emission was 410 nm. At these wavelengths, there was negligible fluorescence from ssb. ATPase assays were performed by charcoal adsorption with [γ-^{32}P]ATP in the same buffer as the fluorescence studies.

Results

Association of ssb with DNA. It has been shown previously that the interaction of recA protein with εDNA causes a rise in the fluorescence of the DNA, which is further increased by the addition of ATP (Silver & Fersht, 1982). ssb protein also causes a rise in εDNA fluorescence. Titration of εDNA with ssb gives a stoichiometry of binding of 10 nucleotides/ssb monomer (Figure 1). This value is in excellent agreement with earlier studies (Kornberg, 1980; Krauss et al., 1981). The total extent of fluorescence change was similar to that on addition of recA. There was no further change in fluorescence, however, on the addition of ATP.

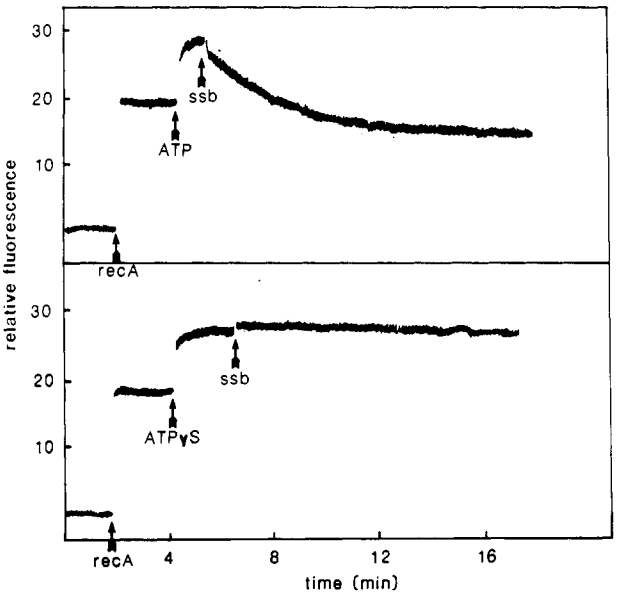

FIGURE 2: Effect of addition of a saturating concentration of ssb (0.4 μM) to the recA–εDNA–NTP complex (containing 0.5 μM recA and 3 μM εDNA): (top) NTP is ATP (1 mM); (bottom) NTP is ATPγS (0.2 mM).

Effect on Fluorescence of recA–ATP–εDNA Complex on Addition of ssb. On the addition of saturating ssb to a preformed complex of recA–εDNA–ATP containing stoichiometric recA (1 mol of recA/6 mol of nucleotide), a fall in the fluorescence was observed (Figure 2). The final extent of fluorescence was slightly lower than that observed for recA and εDNA alone. In the absence of ssb, the fluorescence remained constant for about 1 h, and then a very slow change was observed.

The decrease in fluorescence followed first-order kinetics over three half-lives with $t_{1/2}$ = 3–3.5 min (determined from semilogarithmic plots). The fluorescence at the end point remained constant for several hours.

ATPγS could not substitute for ATP in this reaction. If the recA–εDNA complex was preformed with ATPγS in place of ATP, no change was observed on the addition of ssb (Figure 2). Addition of ATPγS to the reaction mixture during the decrease in fluorescence halted the reaction immediately—no further changes were seen for at least 30 min. For any other order of addition of compounds, no fluorescence changes were observed other than the initial rise caused by binding of protein to εDNA.

Concentration of Free recA in Solution after Fluorescence Changes. The observed decrease in fluorescence could be caused by the formation of a complex between ssb, recA, and εDNA (and ATP) or by the displacement of recA from εDNA by ssb. To distinguish between these possibilities, the concentration of free recA in solution after the fluorescence change was completed was estimated from ATPase measurements after the addition of excess ssDNA. When saturating ssb was added to a preformed complex of recA–ATP–εDNA, the ATPase activity of the recA was inhibited (Figure 3). This again could be attributed to either formation of a ssb–recA–εDNA complex or displacement of recA from the DNA (DNA-independent ATPase activity is much lower than DNA-dependent ATPase activity). The addition of an excess of single-stranded calf thymus DNA to this solution does not apparently affect the fluorescence but should be capable of stimulating the ATPase activity of any free recA in solution. When this was performed with a stoichiometric concentration of recA present, there was about 40% of the activity expected

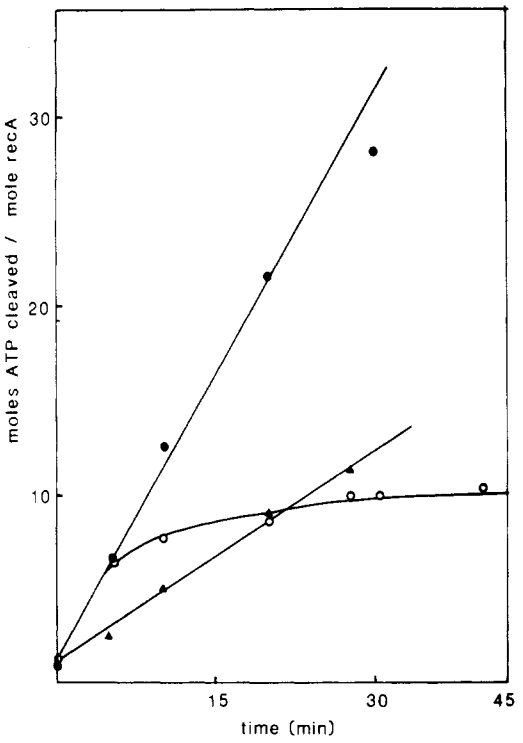

FIGURE 3: Use of ATPase activity measurements to calculate concentration of recA remaining bound to ϵDNA after addition of a saturating concentration of ssb (0.4 μM) to ϵDNA–recA–ATP complex (containing stoichiometric recA; i.e., [recA] = 0.5 μM and [ϵDNA] = 3 μM): (●) presence of 20 μM calf thymus ss DNA; (○) addition of ssb at t = 0; (▲) addition of 20 μM single-stranded calf thymus DNA to a preformed complex of ssb–recA–ϵDNA.

if all the recA was free in solution to bind to calf thymus DNA (Figure 3). The same result was obtained whether the complex had been formed for 20 min or 1 h, suggesting no aging occurred. At ssb concentrations below saturating, there was some residual ATPase in the absence of any added ssDNA.

Reduction of recA concentration below the stoichiometric results in a decrease in the amount of recA protein released into the solution; at a ratio of 11 ϵDNA nucleotides/recA monomer, no recA was released from the complex as judged by the ATPase activity on the addition of excess calf thymus DNA.

The inhibition of the ATPase reaction on the addition of ssb was not immediate but occurred with a half-time of 3.5–4 min. During this time, 8–15 mol of ATP was cleaved per monomer of recA present. Preincubation of ssb with DNA before the addition of recA did not appear to give complex formation, as in this case at least 90% of recA was still free.

Effect of Protein Concentration on Reaction. Both the extent and rate of fluorescence changes were altered by lowering the concentration of ssb (below saturating) while keeping the recA concentration constant (Figure 4). The extent of change observed was linearly related to the concentration of ssb present; i.e., 50% saturation produced 50% reaction. Increasing the concentration had only a slight effect on rate and no effect on extent. If the concentration of ssb was kept constant and the recA concentration changed, very little effect on the rate or extent was observed over a 20-fold range except at very low concentrations (less than saturating) when a slight increase in half-time was observed.

Relative Affinity of ssb for DNA and DNA–recA–ATP Complexes. If small aliquots of ssb are used to titrate a solution of recA–ATP–ϵDNA where the recA concentration is half-saturating, it appears that ssb has a greater affinity for free ϵDNA than the complex. No fall of fluorescence is observed until the remaining half of the DNA has been saturated with ssb (Figure 5). At saturating recA, levels, the drop in fluorescence occurs on addition of the first aliquot of ssb. If ATP is replaced by ATPγS, similar rises in fluorescence are seen until the ϵDNA is saturated, but no decrease is seen on addition of further aliquots.

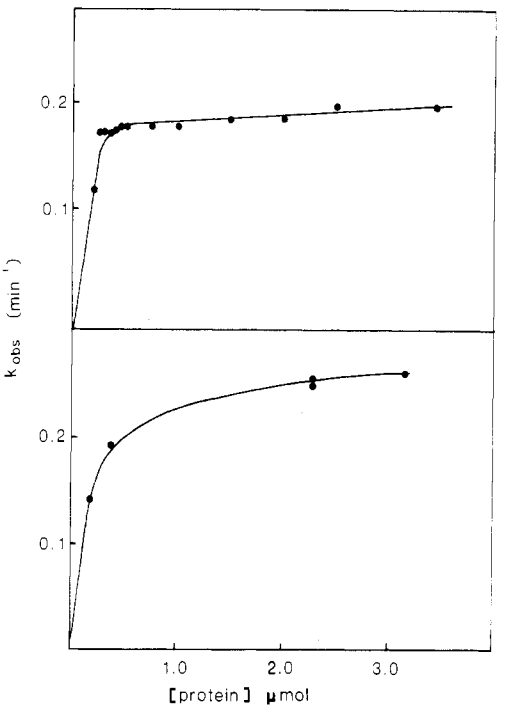

FIGURE 4: Dependence of rate of fluorescence change accompanying formation of recA–ssb–ϵDNA complex on concentrations of ssb and recA ([ϵDNA] = 3 μM): (top) ssb constant (0.4 μM), recA variable; (bottom) recA constant (0.5 μM), ssb variable.

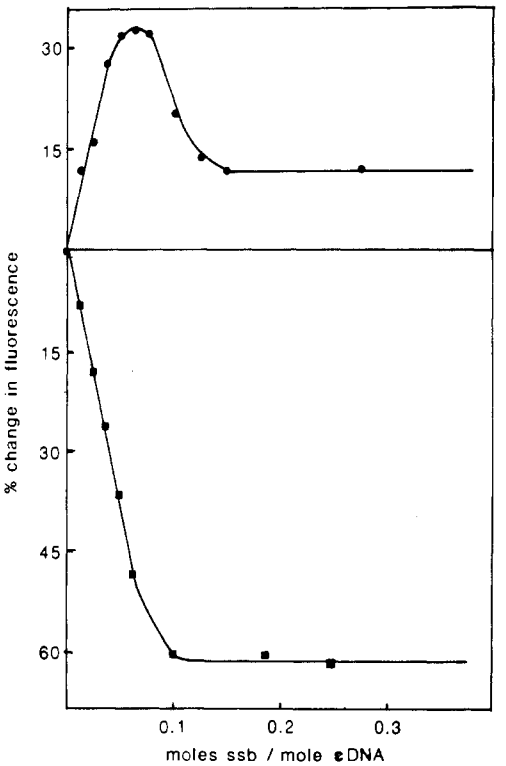

FIGURE 5: Binding of ssb to ϵDNA in the presence of saturating and half-saturating recA concentrations. (●) Titration of ssb with ϵDNA–recA–ATP complex containing half-stoichiometric recA ([recA] = 0.25 μM, [ϵDNA] = 3 μM). (■) Titration of ssb with complex as above but in the presence of saturating recA ([recA] = 1.5 μM, [ϵDNA] = 3 μM).

Effect of Nucleotides on Fluorescence Changes. Lowering the ATP concentration made no difference to either the extent or the rate of the change until the concentration fell below 0.13 mM when an increase in rate was observed (at 8 μM ATP, the $t_{1/2}$ was about 2.5 min compared with the 3.5 usually seen). Addition of a high concentration of ADP also accelerated the decrease of fluorescence. Addition of a similar concentration to the recA–ATP–εDNA complex resulted in some decrease in fluorescence, but the change was very slow (half-reaction was complete in 34 min).

Effect of High Concentration of Salts on Fluorescence Changes. The presence of 150–250 mM NaCl appears to reduce the extent of the reaction slightly (by about 10%) while increasing the rate ($t_{1/2}$ = 1.8–2.1 min). The presence of such high concentration of salt does not appear to affect the binding of ssb to εDNA alone. There is, however, a general lowering of all fluorescence yields observed, perhaps due to some sort of quenching.

Effect of Replacement of Mg^{2+} by Other Divalent Cations. Substitution of Mn^{2+} for Mg^{2+} gives a rise rather than a fall in fluorescence on the addition of ssb, while Ca^{2+} gives a decrease in the fluorescence, although the extent is not as great as that with Mg^{2+} present. In neither case does the presence of a different divalent cation appear to affect significantly the formation of recA or ssb complexes with εDNA alone, although again there seems to be some fluorescence quenching.

Effect of Competing Nonfluorescent Polynucleotides. recA has a very high affinity for poly(dT) (Silver & Fersht, 1982). Addition of poly(dT) at a concentration high enough to cause complete transfer of recA to poly(dT) in the absence of ssb had only a negligible effect on the fluorescence if ssb was present. This relative affinity for εDNA and poly(dT) appeared to be very similar to that for poly(dT) alone. Addition of single-stranded calf thymus DNA even to a large excess had very little effect on the fluorescence of any of the complexes.

Effect of Change in pH. If sodium maleate (pH 6.2) is used in place of Tris-HCl, a similar decrease in fluorescence is observed. However, the rate is much lower: $t_{1/2}$ is 9 min compared with 3.5 under comparable conditions at pH 8.1.

Discussion

The addition of ssb to the εDNA–recA–ATP complex causes a marked decrease in fluorescence. However, it causes only a fraction of the recA to dissociate from the εDNA. This suggests that ssb is interacting directly with the εDNA–recA–ATP complex to give a tertiary complex. As the rate of formation of the complex is slow and the stoichiometry of DNA to recA is increased from 6:1 to 10:1 nucleotides/recA monomer, ssb must be causing a major rearrangement of the recA–εDNA–ATP complex. Formation of the complex seems to require the hydrolysis of ATP since there is no formation in the presence of ATPγS. Also, substitution of Mn^{2+} for Mg^{2+} (when no ATPase occurs, Weinstock et al., 1982) causes no decrease in fluorescence.

There appear to be several turnovers of ATP during the formation of the complex. This probably results from hydrolysis catalyzed by recA–εDNA during the slow rate of formation of the tertiary complex and does not necessarily imply that extensive ATP cleavage is required.

The final complex has no detectable ATPase activity. However, it must be capable of hydrolytic activity under certain conditions since this is required for the strand-exchange reaction (West et al., 1981). There is no tight binding of nucleotides by the ternary complex: preliminary investigations using equilibrium dialysis and filter-binding assays failed to detect tightly bound ATP or ADP within the complex.

Some form of interaction between ssb and recA–ssDNA–ATP complexes has been suggested by other groups (Cox & Lehman, 1982; Resnick & Sussman, 1982). Using εDNA, it is possible to monitor its formation directly and continuously by fluorescence. Further, since the final complex is also fluorescent, changes caused by presence of different cofactors are readily observable. However, like previous studies, it provides no evidence for direct interaction between ssb and recA. It is therefore possible that indirect interactions occur; i.e., the ssb "orders" the DNA in some way, influencing the binding of recA to DNA and possibly its other substrates as well.

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