Kinetic Studies of recA Protein Binding to a Fluorescent Single-Stranded Polynucleotide†

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ABSTRACT: Fluorescence spectroscopy was used to investigate the binding of *Escherichia coli* recA protein to a single-stranded polynucleotide. Poly(deoxy-1,N^6-ethenoadenylic acid) was prepared by reaction of chloroacetaldehyde with poly(deoxyadenylic acid). The fluorescence of poly(deoxy-1,N^6-ethenoadenylic acid) was enhanced upon recA protein binding. The kinetics of the binding process were studied as a function of several parameters: ionic concentration (KCl and $MgCl_2$), pH, nature of the nucleoside triphosphate [adenosine 5′-triphosphate or adenosine 5′-*O*-(γ-thiotriphosphate)], protein and polynucleotide concentrations, polynucleotide chain length, and order of sequential additions. The observed kinetic curves exhibited a lag phase followed by a slow binding process characteristic of a nucleation–elongation mechanism with an additional slow step governing the rate of the association process. The lag phase reflecting the nucleation step was not observed when the protein was first bound to the polynucleotide before addition of adenosine 5′-triphosphate. Adenosine 5′-triphosphate induced a dissociation of the recA protein, which was immediately followed by binding of the recA–adenosine 5′-triphosphate–Mg^{2+} ternary complex. The origin of this "mnemonic effect" and of the different kinetic steps is discussed with respect to protein conformational changes and aggregation phenomena.

The recA gene product of *Escherichia coli* is involved in two major processes of the bacterial life: general genetic recombination and induced DNA repair (Dressler & Potter, 1982; Little & Mount, 1982). In relation to its role in genetic recombination, recA protein catalyzes strand exchange in vitro between two homologous DNA molecules provided that at least one of them possesses a single-strand gap (West et al., 1981, 1982). When bound to single-stranded DNA, recA protein induces the cleavage of lexA protein, which is the common repressor of a set of genes involved in the so-called SOS response (Little et al., 1980). This response is elicited when normal replication is arrested due to a block of the replication fork consecutive to thymidine starvation, inhibition of gyrase activity with nalidixic acid, or lesions of the DNA introduced by exposure of the cell to chemical or physical agents such as carcinogens or UV radiations. In vitro studies have shown that, in addition to single-stranded DNA, two additional factors, ATP[1] and magnesium, are required for recA protein to exert both types of activities: strand exchange and induced proteolytic cleavage of lexA protein. The quaternary recA–ssDNA–ATP–Mg^{2+} complex is thought to play a fundamental role in vivo. A detailed investigation of the different steps involved in its formation is necessary to get a complete understanding of the possible ways by which recA protein regulates its activities. For this purpose we used the property of recA protein to bind to fluorescent derivatives of nucleic acids such as ϵDNA and poly(dϵA) and to enhance their fluorescence quantum yield so that binding of recA protein can be easily monitored by spectrofluorometry (Silver & Fersht, 1982, 1983; Cazenave et al., 1983, 1984; Menetski & Kowalczykowski, 1985). Poly(dϵA), the fluorescent derivative of poly(dA) obtained by reaction with chloroacetaldehyde, has been shown to be very useful in investigating the nucleic acid binding properties of the recA protein (Cazenave et al., 1983, 1984).

Materials and Methods

recA protein and poly(dϵA) were prepared as previously described (Cazenave et al., 1983). The single-strand binding protein from *E. coli* (SSB) was a generous gift from Dr. G. Krauss (Hanover). Creatine kinase, creatine phosphate, snake venom phosphodiesterase from *Crotalus terrificus*, pancreatic DNase, ATP, and ATPγS were purchased from Boehringer Mannheim; poly(dA) and oligodeoxyadenylic acids were from P-L Biochemicals. Chloroacetaldehyde (50–55% in water) was a Fluka product. The number of residues of the oligodeoxyadenylates was determined by electrophoresis on an 8% acrylamide–7 M urea gel after 3′-OH end labeling with [α-^{32}P]dideoxyATP and nucleotidyl transferase. Their molar extinction coefficient was determined by quantitating dϵAMP released by enzymatic hydrolysis of the oligonucleotide chain with pancreatic DNase and snake venom phosphodiesterase. Concentrations of polynucleotides and oligonucleotides are expressed as moles of phosphate per liter. Fluorescence and light scattering measurements were performed on an Aminco SPF 500 spectrofluorometer in 1 × 1 cm quartz cuvettes thermostated at 20 °C. Fluorescence excitation and emission wavelengths were 320 and 410 nm, respectively. Light scattering was measured with both excitation and emission wavelengths at 350 or 410 nm. Reactions were started by mixing the investigated solutions by use of micropipettes. It

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(γ-thiotriphosphate); ϵ DNA, single-stranded DNA whose adenine and cytosine residues have been converted into their etheno derivatives; ssDNA, single-stranded DNA; EDTA, ethylenediaminetetraacetic acid; poly(dA), poly(deoxyadenylic acid); poly(dϵA), poly(deoxy-1,N^6-ethenoadenylic acid); dϵAMP, deoxy-1,N^6-ethenoadenosine 5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; SSB, single-strand binding protein from *E. coli*.

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took about 10 s after mixing before fluorescence could be measured accurately. Therefore no data will be given for times shorter than 10 s. TE buffer contained 20 mM Tris–HCl, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA, whereas TK buffer contained 150 mM KCl in addition to the other components of TE buffer. pH was adjusted to the desired value ranging from 7.0 to 7.5 and will be specified for every experiment. CE buffer contained 20 mM sodium cacodylate (pH 6.2), 1 mM 2-mercaptoethanol, and 0.1 mM EDTA. When used, the ATP-regenerating system consisted of 8 units/mL creatine kinase and 2 mM creatine phosphate.

We observed some differences in the absolute values of the quantitative parameters with different poly(dεA) preparations and aging. However, the relative values and the qualitative features were not altered. Consequently, all comparative experiments were carried out the same day with the same sample of poly(dεA).

RESULTS

General Features of the Kinetics of recA Protein Binding to Poly(dεA). Binding of recA protein to poly(dεA) could be followed by the enhancement of the polynucleotide fluorescence, probably due to the unstacking of the bases induced by stretching the polynucleotide chain (Cazenave et al., 1984). recA protein can bind to poly(dεA) in the absence of both Mg^{2+} and ATP (or ATPγS, an ATP analogue not hydrolyzed by the protein). In the absence of magnesium, addition of ATP or ATPγS induced a dissociation of the recA–poly(dεA) complexes. A similar behavior was previously observed with single-stranded DNA (Cox et al., 1981). Further addition of magnesium after ATP or ATPγS induced a slow reassociation of recA protein to poly(dεA) as monitored by the fluorescence enhancement (Cazenave et al., 1983). The kinetics of fluorescence change could reflect a conformational change of the polynucleotide–protein complex subsequent to the binding of recA protein to the lattice rather than the binding reaction itself. The same experiment was therefore repeated, monitoring light scattering of the solution measured at right angles with respect to excitation in the spectrofluorometer (λ = 410 nm). In the absence of polynucleotide, the light scattering intensity of recA solutions in TE buffer was very low. Addition of polynucleotides induced a slow but important (5-fold) increase in light scattering. Morrical and Cox (1985) previously demonstrated that light scattering intensity was linearly correlated to the amount of complex. Kinetics followed by fluorescence and light scattering led to identical results, within experimental accuracy (data not shown). Variations in fluorescence intensity were not delayed as compared to protein binding. Therefore, the fluorescence increase monitored the formation of poly(dεA)–recA complexes.

The kinetics of recA protein binding to poly(dεA) had a sigmoidal appearance. In the presence of ATP, after the lag phase, the poly(dεA) fluorescence increased up to a plateau according to a monoexponential law and then slowly decreased (Figure 1). When the ATP-regenerating system was added, the slow decrease disappeared and the increase phase could be decomposed into a sum of two exponentials. The relaxation time corresponding to the faster step was identical with the relaxation time of the monoexponential phase observed in the absence of the ATP-regenerating system. It is known that ADP binding leads to dissociation of recA–polynucleotide complexes (Silver & Fersht, 1982). In the absence of the ATP-regenerating system, ATP hydrolysis generated ADP, which induced a dissociation of recA protein and therefore led to a decrease of poly(dεA) fluorescence. When ATP was replaced by ATPγS, the general shape of the kinetics was not altered: a lag phase was followed by a biexponential increase. However, under identical experimental conditions, a 3-fold decrease of the reaction half-time $\tau_{1/2}$ (defined as the time required to reach 50% of the total fluorescence increase) was observed as compared with ATP (data not shown).

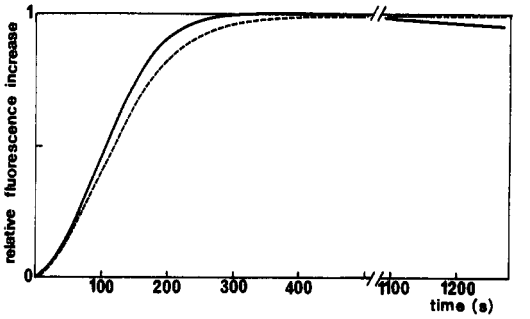

FIGURE 1: Time dependence of the fluorescence change of 3.2 μM poly(dεA) in the presence of 0.32 μM recA protein and 0.7 mM ATP when 4 mM $MgCl_2$ was added to the poly(dεA), recA, and ATP mixture in TE buffer at pH 7.1 in the presence (– –) or in the absence (—) of the ATP-regenerating system.

Table I: Influence of the Order of Sequential Additions on the Shape of Association Kinetics of recA Protein to Poly(dεA)[a]

buffer	nucleotide	last addition	initial state of recA protein	presence of lag phase in binding process
TK	ATP	Mg^{2+}	recA–ATP	+
		poly(dεA)	recA–ATP–Mg^{2+}	+
		ATP	recA–Mg^{2+}	+
		ATP + Mg^{2+}	recA free	+
		recA	recA free	+
	ATPγS	Mg^{2+}	recA–ATPγS	+
		ATPγS	recA–Mg^{2+}	+
TE	ATP	Mg^{2+}	recA–ATP	+
		poly(dεA)	recA–ATP–Mg^{2+}	+
		recA	recA free	+
		ATP	poly(dεA)–recA–Mg^{2+}	no
		ATP + Mg^{2+}	poly(dεA)–recA	no
	ATPγS	Mg^{2+}	recA–ATPγS	+
		poly(dεA)	recA–ATPγS–Mg^{2+}	+
		recA	recA free	+
		ATPγS	recA–poly(dεA)–Mg^{2+}	no
		ATPγS + Mg^{2+}	recA–poly(dεA)	no

[a] Reactions were performed in TE and TK buffers in the presence of 4 mM $MgCl_2$, 3.2 μM poly(dεA), and 0.32 μM recA protein. The experiments in the presence of ATP (1 mM) were carried out at pH 7.0 and those in the presence of ATPγS (0.1 mM) at pH 7.5.

Influence of Sequential Additions. In order to determine what was the limiting step in the binding reaction, the different ligands were added sequentially in different orders. The experiments were performed in TE and TK buffers (Table I). In TE buffer, recA protein bound to poly(dεA) in the absence of nucleotides and magnesium, whereas in TK buffer, whose ionic concentration was higher (150 mM KCl), no binding of recA protein was observed unless both ATP (ATPγS) and Mg^{2+} were simultaneously present. In TK buffer, all kinetics displayed an initial lag phase and were identical whatever the order of the additions. In TE buffer, addition of poly(dεA), Mg^{2+}, or recA protein as the last component led to identical kinetics with a lag phase. On the contrary, when ATP (ATPγS) was added to a poly(dεA)–recA–Mg^{2+} ternary complex, dissociation of the complex was induced, as demonstrated by the rapid initial decrease of the fluorescence

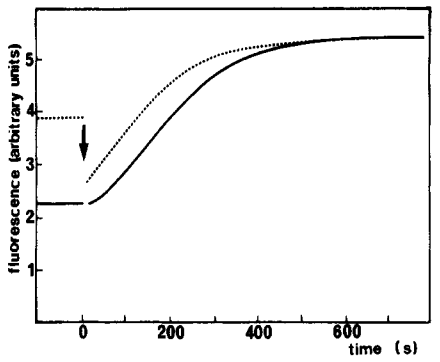

FIGURE 2: Influence of the order of sequential additions on the kinetics of binding of recA protein to poly(dεA). At time 0, Mg^{2+} was added to the poly(dεA), recA, and ATP mixture (—) or ATP to the poly(dεA), recA, and Mg^{2+} mixture (···). Experiments were carried out in TE buffer at pH 7.0 in the presence of 0.32 μM recA protein, 3.2 μM poly(dεA), 1 mM ATP, and 4 mM $MgCl_2$. The arrow indicates that the complex recA–poly(dεA) was first dissociated upon addition of ATP and then followed by binding as a quaternary complex involving recA–Mg^{2+}–ATP and poly(dεA).

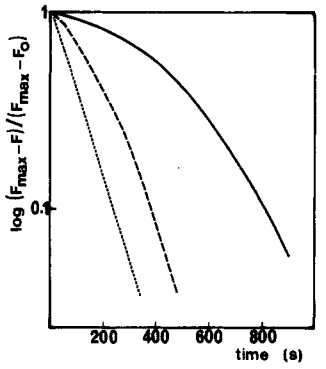

FIGURE 3: Semilogarithmic plot of the time dependence of the fluorescence increase of 3.2 μM poly(dεA) in the presence of 1 mM ATP and 4 mM $MgCl_2$ when successive aliquots of recA protein were added in TE buffer at pH 7.2. Total concentrations of recA protein were 0.16 (—), 0.32 (– –), and 0.48 μM (···). Time 0 corresponds to the addition of each aliquot of recA protein.

intensity (Cazenave et al., 1983), and reassociation of recA protein took place without any lag phase (Figure 2). The same binding curve was obtained when both Mg^{2+} ions and ATP (ATPγS) were added concomitantly to a preformed poly(dεA)–recA complex. These results demonstrated that the recA protein that was being dissociated from the polynucleotide could reassociate to the polynucleotide without a slow intervening step in the minute time range. This property is reminiscent of a mnemonic behavior that was previously demonstrated to occur in several enzymatic processes (Ricard et al., 1974; Papanicolaou et al., 1984).

Successive Additions of recA Protein. As discussed above, a first addition of recA protein to a poly(dεA), ATP, and Mg^{2+} mixture led to kinetics exhibiting a lag phase. However, successive additions of small amounts of a concentrated protein solution resulted in the progressive disappearance of this lag phase (Figure 3). The binding of a few recA molecules on poly(dεA) made further binding of the protein easier. This was typical of cooperative behavior and clearly indicated a mechanism involving nuclation–elongation steps. The single-strand binding protein from *E. coli* (SSB) is known to play an essential role in strand-exchange reactions catalyzed by recA protein (McEntee et al., 1980; Cox et al., 1983) and in the stability of the recA–ssDNA complexes (Cox & Lehman, 1982). We tried to initiate the formation of recA complexes with SSB protein. Binding of the SSB protein to poly(dεA) induced a fluorescence enhancement similar to that induced

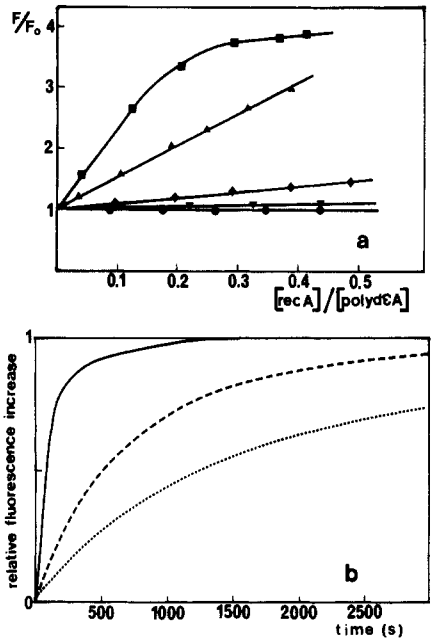

FIGURE 4: Influence of the polynucleotide chain length on recA protein binding. (a) Relative fluorescence intensity of 3.3 μM $(dεA)_8$ (●), 2.6 μM $(dεA)_{12}$ (▼), 3.0 μM $(dεA)_{18}$ (♦), 4.3 μM $(dεA)_{19-60}$ (▲), and 3.7 μM poly(dεA) (■) as a function of increasing recA concentrations in the presence of 4 mM $MgCl_2$ in TE buffer at pH 7.5. (b) Time dependence of the fluorescence increase of 3.3 μM $(dεA)_{18}$ (···), 4.3 μM $(dεA)_{19-60}$ (– –), and 3.8 μM poly(dεA) (—) in the presence of 0.1 mM ATPγS when 4 mM $MgCl_2$ was added to the recA, ATPγS, and polynucleotide mixture in TE buffer at pH 7.5. The concentration of recA protein was 1.6 (··· and – –) or 0.48 μM (—).

by recA protein binding. The kinetics of recA protein binding was not affected by previously bound SSB proteins at a low degree of saturation, indicating that bound SSB could not act as nuclei for recA protein binding (data not shown).

Influence of Polynucleotide Chain Length. Several reports previously noted the importance of polynucleotide chain length on recA protein activity. Short oligonucleotides such as dT_{12} were unable to induce proteolytic or ATPase activity of wild-type recA protein (McEntee & Weinstock, 1981). Competitive binding experiments showed that recA protein did not bind dT_{12} (McEntee et al., 1981). In the presence of ATPγS, stable recA–single-stranded oligodeoxynucleotide complexes were observed with oligonucleotides ranging in size between 9 and 20 residues but not with those 8 residues long (Leahy & Radding, 1986). In order to study the effect of chain length on the kinetics of recA protein binding to a nucleotidic lattice, different oligodeoxyadenylates ranging in length from 8 to 60 residues were modified with chloroacetaldehyde to obtain fluorescent oligo(dεA). These oligo(dεA) displayed spectroscopic properties similar to those of poly(dεA). In all cases, the ratio of optical densities at 300 and 275 nm was equal to 0.57. The molar extinction coefficient at 275 nm of the different oligo(dεA) and poly(dεA) was (3.6 ± 0.1) × 10^3 M^{-1}·cm^{-1} and was very close to that determined by Ledneva et al. (1978) for poly(rεA). The fluorescence intensity was enhanced 12 times upon enzymatic hydrolysis of oligo(dεA) and poly(dεA). This result is in agreement with that previously reported by Tolman et al. (1979) for the dinucleotide εApεA.

As described in Figure 4a, the titration curves of the different oligo(dεA) by recA protein in TE buffer in the presence of Mg^{2+} ions but in the absence of ATP and ATPγS were very dependent upon chain length. No binding was detected in the presence of $(dεA)_8$. A weak binding was observed in the case

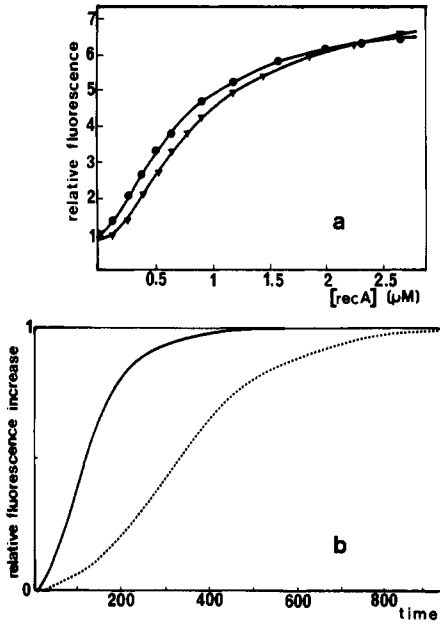

FIGURE 5: Effect of ionic concentration on the binding of recA protein to poly(dεA). (a) Fluorescence intensity of 3.2 μM poly(dεA) as a function of increasing recA concentrations in the presence of 4 mM $MgCl_2$, 1 mM ATP, and the ATP-regenerating system in TE (●) or TK buffer (▼), pH 7.0. (b) Time dependence of the fluorescence increase of 3.2 μM poly(dεA) in the presence of 0.64 μM recA protein and 1 mM ATP when 4 mM $MgCl_2$ was added to the poly(dεA), recA, and ATP mixture in TE (—) or TK buffer (···) at pH 7.0.

of $(d\epsilon A)_{12}$ and $(d\epsilon A)_{18}$. The extent of binding was clearly more important in the case of $(d\epsilon A)_{19-60}$, a mixture of oligonucleotides ranging in length from 19 to 60 residues. However, even for a concentration ratio of recA protein and $(d\epsilon A)_{19-60}$ equal to about 0.5 we failed to observe saturation of the oligonucleotides. The affinity of recA protein for $(d\epsilon A)_{19-60}$ was obviously weaker than for poly(dεA), whose average chain length was about 500 nucleotides. No slow kinetic phenomena were observed under these conditions (absence of ATP or ATPγS). When ATP and Mg^{2+} ions were simultaneously present, recA protein did not bind to any oligonucleotide in both TE and CE buffers. When ATPγS was used instead of ATP, slow binding to the different oligonucleotides was observed except for $(d\epsilon A)_8$. The long time required to observe appreciable binding prevented us from obtaining reliable titration curves. The binding rate increased with chain length: $(d\epsilon A)_{12} < (d\epsilon A)_{18} < (d\epsilon A)_{19-60} <$ poly(dεA) (Figure 4b).

Effect of Ionic Concentration. In TE and TK buffers, the titration curves of poly(dεA) by recA protein obtained in the presence of ATP and of the ATP-regenerating system clearly demonstrated a decrease in the apparent binding constant when ionic concentration increased (Figure 5a). The sigmoidal shape of these curves indicated that recA protein binding to poly(dεA) was cooperative as previously observed with other lattices. An apparent association constant K_{app} could be calculated from the concentration of recA protein required to observe 50% of the fluorescence increase. As previously described (McGhee & von Hippel, 1974), the value of K_{app} is close to $K_i\omega$ for a highly cooperative process, where K_i is the binding constant for an isolated site and ω is the cooperativity parameter. When ATPγS was substituted for ATP, the binding of recA protein to poly(dεA) was much stronger (Cazenave et al., 1984). Accordingly, the titration curves did not show the sigmoidal shape. This allowed us to determine a site size of 4 ± 0.5 nucleotide units for the protein. This value was used to calculate K_{app} for recA protein binding in the presence of ATP. Apparent association constants of 4.0

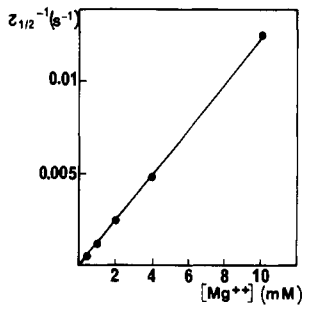

FIGURE 6: Reciprocal of the reaction half-time for the binding of 0.64 μM recA protein to 3.2 μM poly(dεA) as a function of increasing concentrations of $MgCl_2$ in the presence of 0.1 mM ATPγS in TE buffer at pH 7.5.

× 10^6 M^{-1} and 2.2 × 10^6 M^{-1} were obtained in TE and TK buffers (pH 7.0), respectively.

Previous measurements of ω for recA protein binding to εDNA yielded a value of ≈50, independent of ionic concentration (Menetski & Kowalczykowski, 1985). Assuming $K_{app} \approx K_i\omega$, the values of K_i were calculated to be 8.0 × 10^4 M^{-1} and 4.4 × 10^4 M^{-1} in TE and TK buffers, respectively. However, the calculated ω value assumes that only recA monomers bind to poly(dεA). If aggregates of recA proteins bind to the polynucleotide, then ω has to be corrected to take into account the self-association of recA protein (Takahashi et al., 1986). The corrected ω value would be close to 1500 under the experimental conditions used by Menetski and Kowalczykowski. However, there is no experimental evidence that recA aggregates bind to polynucleotides. There is even some indication that this is not a correct assumption (Morrical & Cox, 1985). In the absence of further information on the equilibrium between different aggregated recA species and on their respective binding ability, no attempt was made to interpret the titration curves by using e.g., the equation derived by McGhee and von Hippel (1974) (see Discussion).

The decrease in affinity due to an increase in KCl concentration was concomitant with a decrease in the binding rate (Figure 5b). A similar decrease in the binding rate with salt concentration was observed when ATPγS was used instead of ATP. Addition of 150 mM KCl led to a 3-fold increase in the reaction half-time $\tau_{1/2}$ in the presence of both ATP and ATPγS. The kinetics of formation of stable recA–ssDNA complexes able to perform strand-exchange reactions were previously reported to display a similar dependence with ionic concentration (Soltis & Lehman, 1984).

Influence of Mg^{2+} Concentration. The influence of magnesium concentration was studied in the presence of ATPγS because this nucleotide had a larger affinity for recA protein than ATP (Weinstock et al., 1981). This allowed us to work with lower concentrations in nucleoside triphosphate (0.1 mM instead of 1 mM). Thus we could investigate a wider range of Mg^{2+} concentrations without complications inherent to the presence of nucleoside triphosphates not complexed with divalent cations that could dissociate recA protein from the polynucleotide matrix. Magnesium ions were added to the preincubated poly(dεA), recA, and ATPγS mixture. As illustrated in Figure 6, the reciprocal of the reaction half-time was proportional to the magnesium concentration up to 10 mM $MgCl_2$. No systematic investigations were performed above 10 mM. The rate of recA binding at 20 mM $MgCl_2$ was nearly identical with that observed at 10 mM. Magnesium could act at different levels. It might alter the structure of the polynucleotide and/or the protein–protein interactions. Mg^{2+} also binds to ATP (ATPγS) and might act through the nucleotide–Mg^{2+} complex. The fluorescence quantum yield

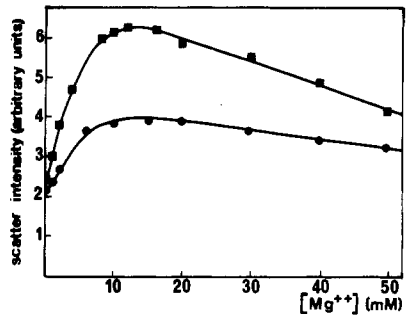

FIGURE 7: Light scattering intensity at 350 nm of a solution containing 0.64 μM recA protein as a function of increasing concentrations of $MgCl_2$ in the presence (■) or the absence (●) of 0.1 mM ATPγS in TE buffer at pH 7.5.

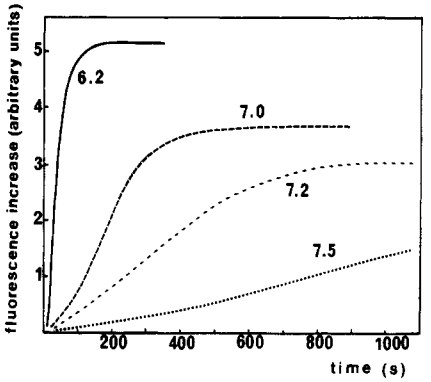

FIGURE 8: Effect of pH on the kinetics of binding of recA protein to poly(dϵA): time dependence of the fluorescence increase of 3.2 μM poly(dϵA) in the presence of 0.32 μM recA protein and 1 mM ATP when 4 mM $MgCl_2$ was added to the poly(dϵA), recA, and ATP mixture in TE buffer at pH 7.5 (···), pH 7.2 (- - -), and pH 7.0 (– –) and in CE buffer at pH 6.2 (—).

of poly(dϵA) increased with Mg^{2+} concentration. Addition of 4 mM $MgCl_2$ induced a 30% increase of poly(dϵA) fluorescence. A 2-fold increase was observed in the presence of 10 mM $MgCl_2$ (data not shown). The light scattering of a recA protein solution was dependent upon magnesium concentration and reached a maximum for a concentration equal to about 15 mM (Figure 7). Our experiments were performed in the presence of 20 mM Tris. Tris concentration has a dramatic influence on the aggregation state of recA protein. Formation of long rodlike filaments was observed in 10 mM Tris but not in 20 mM Tris (Morrical & Cox, 1985). This might explain the discrepancy between our results and those of Cotteril and Fersht (1983). In 10 mM Tris, these authors observed a threshold in magnesium concentration above which light scattering increased sharply. This phenomenon corresponded to the formation of long filaments that were not observed under our experimental conditions. However, light scattering experiments clearly revealed different oligomeric structures of recA protein depending on the experimental conditions. The large effect of Mg^{2+} concentration on the overall binding rate points to a key role of this cation in a crucial step involved in the binding process.

pH Dependence. As illustrated in Figure 8, the pH of the buffer had a dramatic effect on the kinetics of recA protein binding to poly(dϵA). Increase in pH induced a slowing down of the binding rate. In TE buffer, the reaction half-time decreased from 800 s at pH 7.5 to 180 s at pH 7.0. In CE buffer (pH 6.2), the reaction half-time further decreased to 30 s. However,the chemical nature of the ions could play a role in the binding reaction in addition to the pH effect. Therefore, the results obtained in two different buffers cannot be directly compared. The rate of recA protein binding to

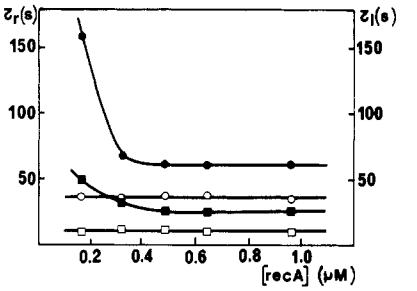

FIGURE 9: Effect of protein concentration on the kinetics of binding of recA protein to poly(dϵA): lag time τ_l (■, □) and relaxation time τ_r (●, ○) for the binding of recA protein to 9.6 μM poly(dϵA) in the presence of 4 mM $MgCl_2$ and either 1 mM ATP in TE buffer at pH 7.2 (closed symbols) or 0.2 mM ATPγS in TE buffer at pH 7.5 (open symbols).

$(d\epsilon A)_{18}$ increased when the pH of TE buffer decreased (data not shown). The affinity of recA protein for poly(dϵA) was also pH dependent with an increase in the apparent association constant when pH decreased from 7.5 to 7.0 (data not shown). However, the slow rate of the binding process at pH 7.5 prevented us from obtaining reliable titration curves and made it difficult to obtain quantitative information on the recA protein binding constant as a function of pH. Previous studies reported pH effects on the affinity of recA protein for double-stranded DNA (McEntee et al., 1981; Cazenave et al., 1984) and on the aggregation of the protein (Kuramitsu et al., 1981; Morrical & Cox, 1985). Therefore, pH variations might play a crucial role on the observed binding rates through an effect on the oligomerization of recA protein (see Discussion).

Influence of Poly(dϵA) and recA Protein Concentrations. An analysis of the concentration dependence of kinetic parameters may indicate the number and the nature of the successive reaction steps. The slow final phase of the fluorescence enhancement was dependent on the final binding density of poly(dϵA). In the case of ATPγS, its amplitude varied from 10% at about 6% saturation of the lattice to 40% when the saturation of the lattice was reached. This phase might be due to effects of excluded site binding (where rearrangement of bound proteins is a prerequisite to further binding) or to binding to shorter poly(dϵA) chains (see above). These two possibilities are not mutually exclusive. Most of our measurements were conducted with an excess of polynucleotide in order to reduce the complications inherent to high binding densities. Due to its small amplitude under such conditions, the slow phase was not further characterized. Measurements were performed in the absence of the ATP-regenerating system since the relaxation time of the apparent monoexponential phase observed under such conditions was identical with the relaxation time of the fast increase observed in the presence of the ATP-regenerating system. Reactions were characterized by the lag time τ_l (defined as time required to reach 5% of the total fluorescence increase) and by the relaxation time τ_r of the fast exponential fluorescence increase. Under our experimental conditions, in the presence of ATPγS, τ_l and τ_r remained practically constant (around 11 and 37 s, respectively) when recA concentration was varied from 0.16 to 0.96 μM (Figure 9). When ATP was used instead of ATPγS, τ_l and τ_r first decreased with increasing recA concentrations up to 0.4 μM and then remained constant and equal to about 27 and 62 s, respectively (Figure 9).

When poly(dϵA) concentration was increased from 1.6 to 12.8 μM at constant recA concentration (0.32 μM), τ_l and τ_r decreased until a plateau was reached (Figure 10). In the presence of ATP, the limit values of τ_l and τ_r were around 37

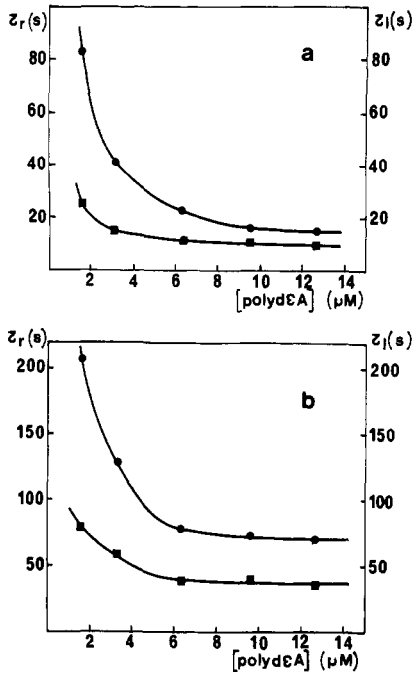

FIGURE 10: Effect of polynucleotide concentration on the kinetics of binding of recA protein to poly(dεA): lag time τ_l (■) and relaxation time τ_r (●) for the binding of 0.32 μM recA protein to poly(dεA) in the presence of 4 mM $MgCl_2$ and either 0.2 mM ATPγS in TE buffer at pH 7.5 (a) or 1 mM ATP in TE buffer at pH 7.2 (b).

and 70 s, respectively, while in the presence of ATPγS, corresponding values were 9 and 15 s.

Discussion

The results presented above describe the binding of the recA protein from *E. coli* to poly(dεA). This fluorescent polynucleotide is an artificial matrix for this protein and might not reflect the binding to a natural single-stranded DNA. However, competition experiments showed that the affinities of recA protein for poly(dεA) and single-stranded DNA were very similar and both polynucleotides stimulated the ATPase activity of recA protein in a similar way (Cazenave et al., 1983, 1984). Formation of presynaptic recA–single-stranded DNA complexes occurred on the same time scale as recA protein binding to poly(dεA) (Kahn & Radding, 1984; Soltis & Lehman, 1984). Therefore, the fluorescence properties of poly(dεA) appeared to be a convenient tool to obtain information on recA protein binding to DNA. The sigmoidal shape of the equilibrium binding curves and the dependence of affinity upon polynucleotide chain length suggest that the affinity of recA protein for isolated sites is relatively low and that stabilization by cooperative contacts is required to observe an appreciable extent of binding. This should be reflected in the binding kinetics by a nucleation–elongation mechanism. According to such a mechanism, the isolated complexes NP_{is}, although present only in limited quantity due to the low affinity of protein P for the isolated sites of the polynucleotide N, serve as nucleation sites for the association of further protein molecules. The binding of another protein close to NP_{is} involves stabilization of this complex via cooperativity and creates the starting chain from which the protein polymerizes (NP_{pol}). This mechanism may be represented as

$$N + P \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} NP_{is} \xrightarrow[k_p]{P} NP_{pol} \qquad (1)$$

Some of our kinetic results are in agreement with such a model: (i) Successive additions of recA protein to a poly(dεA) solution led to a gradual disappearance of the lag phase. This

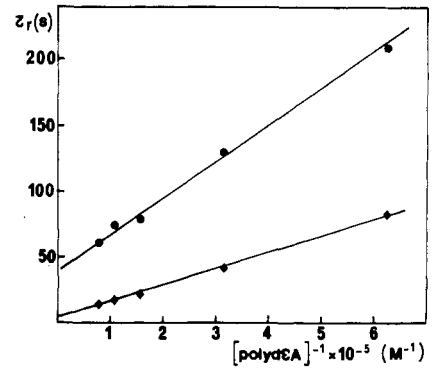

FIGURE 11: Relaxation time τ_r vs. the reciprocal of total polynucleotide concentration for the binding of recA protein to poly(dεA) in the presence of ATP (●) or ATPγS (♦) under the conditions of Figure 10.

situation was reminiscent of the disappearance of the lag phase observed when sonicated actin filaments were added to G-actin (Korn, 1982) and suggested that formation of the first recA–poly(dεA) complexes acted as nuclei promoting further binding of recA protein.

(ii) The rate of recA protein binding to poly(dεA) increased with polynucleotide chain length. When isolated complexes are stable enough and do not require stabilization via cooperativity, the rate constant is expected to decrease with increasing chain length at a constant total polynucleotide concentration due to a change in the cross section for "capture" of the protein by the polynucleotide chain (Lohman & Kowalczykowski, 1981).

(iii) Increase in ionic concentration induced both a decrease in the apparent affinity of recA protein for poly(dεA) and a slowing down of the binding reaction. If it is assumed that the cooperativity parameter ω does not depend on ionic concentration (Menetski & Kowalczykowski, 1985), the observed decrease in the apparent affinity implies a decrease in the number of isolated complexes and thus in the elongation rate. Along the same line, substitution of ATP by ATPγS induced both an increase in the apparent affinity of recA protein for poly(dεA) and an acceleration of the binding.

All these experimental observations were in agreement with a nucleation–elongation mechanism. We therefore attempted to analyze our data according to the assumption that the first step of eq 1 was fast as compared to the second one and could be treated as a preequilibrium. Such a mechanism was found to apply to several DNA-binding proteins such as gene 32 protein from phage T4 (Lohman & Kowalczykowski, 1981) and gene 5 protein from phage fd (Porschke & Rauh, 1983). According to eq 1 under the assumption that τ_r^{-1} is the apparent rate constant for the formation of NP_{pol}, a plot of τ_r vs. the reciprocal of total polynucleotide concentration should be linear (Lohman & Kowalczykowski, 1981)

$$\tau_r = (1/k_p[P]_t)(1 + 1/K_i[N]_t) \qquad (2)$$

with $[P]_t$ and $[N]_t$ the total concentration of protein and polynucleotide, respectively, $K_i = k_i/k_{-i}$ the equilibrium constant for the isolated sites, and k_p the rate constant for the elongation process. This linear dependence was observed when we analyzed the kinetics obtained at various poly(dεA) concentrations (Figure 11). From this plot we could determine K_i and k_p. We found K_i equal to 4×10^4 M^{-1} and 1.7×10^5 M^{-1} and k_p equal to 7×10^5 $M^{-1} \cdot s^{-1}$ and 7×10^4 $M^{-1} \cdot s^{-1}$ in TE buffer, in the presence of ATPγS (pH 7.5) and ATP (pH 7.2), respectively, with polynucleotide concentration expressed as moles of nucleotides per liter. Plots of τ_r^{-1} vs. the concentration of isolated complexes NP_{is} calculated from the

values of K_i were linear (results not shown). Their slope led to values of k_p identical with those determined according to eq 2. With an average length of 500 nucleotides per poly(dεA) chain, the rate constants k_p can be recalculated as 3.5×10^8 M^{-1} (polynucleotide)·s^{-1} and 3.5×10^7 M^{-1} (polynucleotide)·s^{-1} in the presence of ATPγS and ATP, respectively. These values of k_p are low as compared to the values expected for a diffusion-controlled process. Assuming a monomeric state for the free protein, a modified von Smoluchowski equation (Berg et al., 1981) led to an estimate of rate constant k_p of 2×10^9 M^{-1} (polynucleotide)·s^{-1}.

The observed dependence of the binding rate constant vs. poly(dεA) concentration agreed with a model in which a preequilibrium preceded an elongation step although this step might not be diffusion controlled. However, in the case of eq 1, the elongation phase should be a pseudo-first-order reaction with respect to the protein concentration, with an apparent rate constant τ_r^{-1} increasing linearly with total protein concentration (Lohman & Kowalczykowski, 1981). This is in contrast with what was experimentally observed (Figure 9). In the presence of ATPγS, τ_r^{-1} did not vary with recA protein concentration. In the presence of ATP, after an initial increase, τ_r^{-1} reached a plateau and did not change anymore with increasing recA concentration. In order to explain these results, at least one additional rate-limiting step has to be added before the polynucleotide binding step. The similarity in the variations of τ_1 and τ_r vs. recA and poly(dεA) concentrations argues that this slow step is reflected in both the nucleation and elongation rates. This "activation" step might be a slow conformational change of recA protein (eq 3) or a dissociation of recA aggregates (eq 4):

$$P \rightleftarrows P^* \overset{+N}{\rightleftharpoons} NP_{is} \xrightarrow{P^*} NP_{pol} \quad (3)$$

$$P_n \rightleftarrows P^* \overset{+N}{\rightleftharpoons} NP_{is} \xrightarrow{P^*} NP_{pol} \quad (4)$$

In these equations, P represents the complex of recA protein with ATP (ATPγS) and Mg^{2+}. The equilibrium constant of the first step has to be very small so that the concentration of the binding species P* is very low. Both nucleation and elongation require a slow displacement of the equilibrium from the major species P_n or P to the "activated" species P*. Formation of P or P_n from free recA protein not complexed to Mg^{2+} and ATP (ATPγS) is fast as compared to the first step of eq 3 and 4 since identical kinetics were observed whatever the order of the sequential additions, so long as recA protein was not initially bound to poly(dεA) (see below).

The mechanism depicted in eq 3 and 4 might explain qualitatively the behavior of the rate constants with increasing recA and poly(dεA) concentrations (Figures 9 and 10). If recA and poly(dεA) concentrations are small enough, the rate of polymerization is limited by the concentration of nuclei that is proportional to initial poly(dεA) and recA concentrations. When poly(dεA) or recA concentration increases, the displacement of the first equilibrium from the nonbinding to the binding species becomes the limiting step in the overall polymerization process. Therefore, the rate constant should reach a limit value as experimentally observed.

The striking dependence of the global rate with Mg^{2+} concentration (Figure 6) might be explained by a role of this ion in the "activation" process. The binding of a magnesium ion to a site of low affinity of P* (K_d in the range of 10 mM) might be required for the subsequent binding of recA protein to the polynucleotide. A similar situation was previously observed for actin. The binding of a first Mg^{2+} ion to G-actin (K_d = 30 μM) induced a conformational change. Then binding of a second magnesium ion (K_d = 5 mM) led to a species capable of polymerization (Frieden, 1983). The formation of the recA species that polymerizes on the polynucleotide (P*) might also result from a conformational change due to binding of a magnesium ion to a low affinity site of P. However, we cannot exclude that Mg^{2+} ions also act in the polymerization process by modifying the structure of the polynucleotide or that binding to several nonspecific sites of low affinity on the protein induces a screening effect and reduces electrostatic repulsion between the protein and the polynucleotide. Mg^{2+} ions affect the aggregation state of the protein (Figure 7), but nothing is known about the influence of Mg^{2+} concentration on the kinetics of aggregation.

These data do not allow us to specify whether the observed rate-limiting process is a conformational change or dissociation of protein aggregates. There are several examples in the literature where slow protein isomerization has been described. For instance, actin monomer undergoes a conformational change in the second time range following magnesium binding, which is a prerequisite to polymerization (Frieden, 1983). In favor of the last mechanism (dissociation of aggregates prior to nucleic acid binding), it was previously demonstrated that recA protein formed highly aggregated structures in aqueous solutions that might have to dissociate before binding (McEntee et al., 1981; Kuramitsu et al., 1981; Flory & Radding, 1982). It should be noted that a pH increase, which is known to induce a greater oligomerization of the protein, also induced a slowing down of the kinetics of recA protein binding to poly(dεA) and $(d\epsilon A)_{18}$. It is quite possible that either the monomer or an oligomer of defined size (dimer or tetramer, for example) is the nucleic acid binding species. Formation of tubulin oligomers was recently reported to slow down the rate of microtubule nucleation and the net rate of self-assembly (Murphy & Wallis, 1985, 1986).

The lag phase disappeared when recA protein was initially bound to the polynucleotide in the absence of ATP (ATPγS) and then dissociated and reassociated upon addition of ATP (ATPγS) (Table I; Figure 2). This might be explained if the backward rate of the first step of eq 3 and 4 is slow with respect to the second step. The dissociated protein would not be in an equilibrium state but in a conformation or aggregation state well adapted for immediate rebinding. Such a phenomenon might be interpreted as a mnemonic effect of the protein. However, we cannot rule out the hypothesis that some protein oligomers were not dissociated from the polynucleotide upon addition of ATP (ATPγS) and acted as nuclei promoting fast binding of the protein. Further experiments are clearly needed before a definite conclusion can be reached.

A complete analysis of recA protein binding to polynucleotides requires a knowledge of the different species that are present in solution and of their respective ability to bind a polynucleotide chain. The aggregation state of recA protein is very sensitive to external conditions (ionic concentration, chemical nature of both anions and cations, pH, etc.). Applying, e.g., the McGhee–von Hippel equation for cooperative binding to such a system might yield misleading parameters if the binding species is not well-defined and if the equilibrium constants between the different species coexisting in solution are not fully characterized. In the absence of such information, it is not possible to obtain a quantitative picture of recA protein binding to nucleic acids.

The overall rate of formation of the biologically active recA–polynucleotide complexes is dramatically slow as compared to other cooperative systems such as gene 32 protein from phage T4 or gene 5 protein from phage fd for which the

association rates via a nucleation–elongation mechanism are in the range 10 ms–1 s (Lohman & Kowalczykowski, 1981; Porschke & Rauh, 1983). The slow initial process required in the case of recA protein might be involved in vivo to delay the SOS response when a replication fork is blocked by imposing a lag before binding to single-stranded DNA. This lag would allow the "housekeeping" repair enzymes to try to eliminate DNA damages, the SOS response being delayed and induced only if these enzymatic activities become a limiting factor. Then further binding of recA protein would be accelerated if the protein keeps memory of previous DNA binding.

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