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Properties of the High-Affinity Single-Stranded DNA Binding State of the *Escherichia coli* RecA Protein†

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ABSTRACT: The properties of the high-affinity single-stranded DNA (ssDNA) binding state of *Escherichia coli* recA protein have been studied. We find that all of the nucleoside triphosphates that are hydrolyzed by recA protein induce a high-affinity ssDNA binding state. The effect of ATP binding to recA protein was partially separated from the ATP hydrolytic event by substituting calcium chloride for magnesium chloride in the binding buffer. Under these conditions, the rate of ATP hydrolysis is greatly inhibited. ATP increases the affinity of recA protein for ssDNA in a concentration-dependent manner in the presence of both calcium and magnesium chloride with apparent K_d values of 375 and 500 μM ATP, respectively. Under nonhydrolytic conditions, the molar ratio of ATP to ADP has an effect on the recA protein ssDNA binding affinity. Over an ATP/ADP molar ratio of 2–3, the affinity of recA protein for ssDNA shifts cooperatively from a low- to a high-affinity state.

The recA protein has been shown to bind to single-stranded DNA (ssDNA)[1] (McEntee et al., 1981; Silver & Fersht, 1982; Menetski & Kowalczykowski, 1985). The affinity of recA protein for ssDNA is influenced by the presence of ATP, ADP, and NaCl (Menetski & Kowalczykowski, 1985). Increasing the concentration of ADP and NaCl decreases the affinity, while the presence of ATP increases the affinity of recA protein for ssDNA. Weinstock et al. (1981) have shown that recA protein can hydrolyze other nucleoside triphosphates in addition to ATP. In this paper, we have extended our previous analysis of the modulation of recA protein ssDNA binding affinity by nucleotide cofactors to include those nucleotides having significant concentrations in vivo. The data show that all nucleotides which are hydrolyzed by recA protein induce a high ssDNA binding affinity state similar to that induced by ATP.

The ATP-induced high-affinity state was first observed (Silver & Fersht, 1982) and characterized (Menetski & Kowalczykowski, 1985) under conditions which support the ATPase activity of recA protein. Therefore, the data reflect only the steady-state effect of ATP on recA protein ssDNA binding affinity. Under these conditions, the individual effects of binding and hydrolysis of ATP cannot be clearly separated. Ideally, the two events could be separated under conditions that prevent hydrolysis but not nucleotide binding. Weinstock et al. (1981) have reported that in the presence of $CaCl_2$, the rate of ATP hydrolysis is less than 10% of that observed in $MgCl_2$. We have found that ATP is indeed bound to recA protein in the presence of $CaCl_2$ and that ATP induces a high ssDNA binding affinity state similar to the one observed in $MgCl_2$. This result shows that only ATP binding, and not hydrolysis, is required to induce the high-affinity state. Under conditions of reduced hydrolysis, the affinity of all recA protein molecules for ssDNA is sensitive to the ATP/ADP molar ratio in the buffer. As the ATP/ADP molar ratio decreases, the

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[1] Abbreviations: etheno M13 DNA, modified ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytosine; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); poly(dA), poly(deoxyadenylic acid); RFI, relative fluorescence increase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SSB protein, *E. coli* single-stranded DNA binding protein.

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affinity of the bound recA protein changes abruptly from a high to a low ssDNA affinity state. These data show that under these conditions, in the presence of nucleotide cofactors, recA protein exists exclusively in one of two affinity states.

Materials and Methods

RecA Protein. RecA protein was purified from *Escherichia coli* strain JC12772 (Uhlin & Clark, 1981) by using a preparative protocol (in preparation) based on spermidine precipitation (Griffith & Shores, 1985). Protein concentrations were determined by using an extinction coefficient of 2.7×10^4 cm^{-1} M^{-1} at 280 nm.

Chemicals. ATP, ADP, and ATPγS were purchased from Boehringer Mannheim and were dissolved as concentrated stock solutions at pH 7.5. Concentrations of adenine nucleotides were determined spectrophotometrically by using an extinction coefficient of 1.54×10^4 M^{-1} cm^{-1} at 260 nm. ATPγS contained less than 5% ADP and was used without further purification. The remaining nucleoside triphosphates and diphosphates were purchased from Sigma Chemical Co. All nucleotides were dissolved as concentrated stock solutions at pH 7.5 and were used within several days. The concentration of each nucleotide was determined spectrophotometrically using the following extinction coefficients: dATP and dADP, $\epsilon = 1.54 \times 10^4$ cm^{-1} M^{-1} at 260 nm; ribo- and deoxy-CTP and -CDP, $\epsilon = 9.1 \times 10^3$ cm^{-1} M^{-1} at 271 nm; ribo- and deoxy-GTP and -GDP $\epsilon = 1.19 \times 10^4$ cm^{-1} M^{-1} at 258 nm; TTP, UTP, TDP, and UDP, $\epsilon = 8.1 \times 10^3$ cm^{-1} M^{-1} at 261 nm. Poly(deoxyadenylic acid) was purchased from P-L Biochemicals and used as a concentrated stock solution. The concentration of poly(dA) was determined by using an extinction coefficient of 8600 cm^{-1} M^{-1} at 259 nm.

Etheno-Modified M13 ssDNA. Etheno M13 DNA was made as described by Menetski and Kowalczykowski (1985). The concentration of etheno M13 DNA was determined by using an extinction coefficient of 7.0×10^3 cm^{-1} M^{-1} at 260 nm (Menetski & Kowalczykowski, 1987).

Fluorometric Binding Assay. Binding assays were conducted in standard binding buffer (20 mM Tris-HCl, pH 7.5, and 0.1 mM dithiothreitol) containing various concentrations of $MgCl_2$ or $CaCl_2$, as indicated. Salt titration and protein titration experiments were conducted at either 25 or 37 °C, as indicated. Changes in the fluorescence of the etheno M13 DNA, due to recA protein binding, were monitored with a Perkin-Elmer MPF-44E fluorescence spectrophotometer and recorded with a Hewlett-Packard 3421A data acquisition unit interfaced to a Hewlett-Packard 85B computer. Aliquots of concentrated salt (for salt titrations) or protein solutions (for forward protein titrations) were added by using a Hamilton Micro-lab P programmable pipettor under computer control. Increases in the fluorescence of the etheno M13 DNA upon recA protein binding are reported as a relative increase above the fluorescence of the uncomplexed recA protein and etheno M13 DNA. This ratio is determined by dividing the fluorescence of the protein–DNA complex before a salt titration by the fluorescence of the free etheno M13 DNA and recA protein at the end of the salt titration (after correction for dilution). The value for this relative fluorescence increase (RFI) is approximately 1.8 for the complex in the absence or presence of ADP and 2.2 for the complex in the presence of ATP.

During the course of these experiments, we observed that the concentration of phosphoenolpyruvate affected the apparent salt titration midpoint, and thus the affinity of recA protein for ssDNA, in both the absence and the presence of ATP. This effect appears to be competitive with ATP concentration. Concentrations of phosphoenolpyruvate more than 10-fold greater than that of ATP concentration decreased the observed salt titration midpoint (data not shown). Therefore, in all of the experiments described below, the concentration of phosphoenolpyruvate was always less than or equal to 10 times the concentration of ATP.

We have found that under certain conditions the protein titrations described above yield unreliable results. Factors which stimulate the gross aggregation of free recA protein, such as high magnesium and calcium ion concentrations, interfere with the analysis of the intrinsic binding constant and cooperativity. Since the scattering of free protein observed under these conditions is not a linear function of the protein concentration, it is very difficult to subtract this protein scattering effect from the raw data. Consequently, such data cannot be fit to determine K and ω.

Assay for the Hydrolysis of Nucleoside Triphosphate. Hydrolysis of nucleoside triphosphate was assayed by determining the increase in concentration of inorganic phosphate with time, using the method of Lanzetta et al. (1979). Nucleotide hydrolysis assays were conducted under the same buffer conditions as the binding assays to allow direct comparison. Standard assays used 4.7 μM etheno M13 DNA and 0.56 μM recA protein in standard binding buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, and 4 mM $MgCl_2$) at 37 °C. Aliquots of the assay mixture were removed, and the reaction was stopped in 1 N HCl. Early time points in these reactions were linear and were used to determine the initial rate of ATP hydrolysis. Under these conditions, the initial rate of hydrolysis showed a sigmoidal dependence on nucleotide concentration for all nucleotides that were hydrolyzed. Therefore, the apparent K_m values were determined by interpolation of the nucleotide dependence to yield the concentration of nucleotide which induces half of the maximum rate of hydrolysis.

Results

High-Affinity ssDNA Binding State of RecA Protein Can Be Induced by All Nucleoside Triphosphates That Are Hydrolyzed. RecA protein has been shown to hydrolyze nucleoside triphosphates other than ATP, including dATP, UTP, and CTP (Weinstock, 1982). We have extended the analysis of Weinstock (1982) to include the values for the kinetic parameters, K_m and V_{max}, for each nucleotide and also the effect of each nucleotide on recA protein ssDNA binding affinity. The rate of hydrolysis for each nucleoside triphosphate shows a sigmoidal dependence on nucleotide concentration (data not shown) similar to the cooperative dependence observed with respect to ATP concentration (Weinstock et al., 1981; Kowalczykowski, 1986; Menetski & Kowalczykowski, 1987). Thus, the apparent K_m values reported represent the concentration of nucleotide required to induce half of the maximum hydrolysis rate. The apparent K_m values, in Table I, for each nucleotide can differ by more than 20-fold. However, the maximum rates of hydrolysis for each nucleotide, V_{max} (or k_{cat}), differ by less than 4-fold. These data suggest that the major difference in the hydrolysis characteristics of these nucleotides is the binding of nucleotide to form an active hydrolytic complex. Once this complex is formed, the nucleotide is hydrolyzed at a rate comparable to that of ATP.

The apparent affinity of each recA protein–NTP complex for ssDNA was measured to determine whether changes in ssDNA affinity were related to hydrolytic activity. Previous studies have shown that the relative affinity of recA protein for ssDNA is related to the salt concentration required to

Table I: Comparison of RecA Protein NTP Hydrolysis and ssDNA Binding Characteristics with Different Nucleotides

NTP	K_m[a] (μM)	k_{cat} (min^{-1})	ST_{mp}[b] (mM NaCl)	RFI[c]
dATP	50	37.0	450	2.55
ATP	60	26.6	420	2.25
UTP	350	24.3	390	2.13
dCTP	660	20.1	400	2.55
CTP	1120	11.6	415	2.16
NC			270	1.8
GTP	ND[d]	ND	110	1.75
dGTP	ND	ND	85	1.3
TTP	ND	ND	80	1.91

[a] NTP hydrolysis experiments were done with 4.7 μM etheno M13 DNA and 0.56 μM recA protein as described under Materials and Methods at 37 °C in 4 mM $MgCl_2$. All of the nucleotides that induced hydrolysis showed a sigmoidal dependence on nucleotide concentration. The values of K_m were determined as described under Materials and Methods. [b] Salt titrations were done with 5.4 μM etheno M13 DNA and 1.57 μM recA protein as described under Materials and Methods in 4 mM $MgCl_2$. The concentration of NTP used was determined as 10 times the K_m value for that nucleotide; for GTP, dGTP, and TTP, the NTP concentration was 9 mM. [c] Relative fluorescence increase of the etheno M13 DNA upon binding of recA protein in the presence of various nucleoside triphosphates (see Materials and Methods for details). [d] ND, not detectable.

half-dissociate the protein–DNA complex (Menetski & Kowalczykowski, 1985). Salt titration midpoints (ST_{mp}) in the presence of each NTP are also shown in Table I. The data demonstrate that protein–DNA complexes formed with any of the nucleoside triphosphates that are hydrolyzed are stable to high concentrations of NaCl ($ST_{mp} \approx 400$ mM) while those formed with nucleoside triphosphates that are not hydrolyzed are disrupted by low NaCl concentrations ($ST_{mp} \approx 100$ mM NaCl).

The ATP-induced high ssDNA affinity state of recA protein has an experimental characteristic that can be observed when etheno M13 DNA is used. The fluorescence of the etheno M13 DNA increases upon recA protein binding and is even greater in the presence of ATP than in its absence (Silver & Fersht, 1982; Menetski & Kowalcykowski, 1985). This characteristic increase in fluorescence of the protein–DNA complex relative to the fluorescence of the dissociated complex is defined as the relative fluorescence increase (RFI). The relative fluorescence increase observed upon recA protein binding to etheno M13 DNA in the presence of all the nucleotides is also shown in Table I. The fluorescence of the etheno M13 DNA increases 1.8-fold upon the binding of recA protein in the absence of any nucleotide. However, binding of the recA protein–ATP complex to etheno M13 DNA increases its fluorescence 2.3-fold. This greater increase in the fluorescence of the etheno M13 DNA upon recA protein binding is observed only in the presence of the nucleoside triphosphates that can be hydrolyzed. In the presence of the nucleotide triphosphates that are not hydrolyzed, a fluorescence increase similar to that obtained in the absence of nucleotide (1.8) is observed. These data suggest that nucleoside triphosphates which can be hydrolyzed by recA protein can induce a high ssDNA affinity state and a higher relative fluorescence increase.

Nucleoside Diphosphates Reduce the Affinity of RecA Protein for ssDNA. The affinity of recA protein for ssDNA has been shown to decrease in the presence of ADP (Menetski & Kowalczykowski, 1985). Since the above data show that nucleoside triphosphates other than ATP can increase the ssDNA binding affinity of recA protein, the salt titration method was used to determine whether other nucleoside diphosphates could decrease the affinity of recA protein for ssDNA. The salt titration midpoints obtained for various recA protein–nucleoside diphosphate complexes are shown in Table II. All of the nucleoside diphosphates tested decreased the affinity of recA protein for ssDNA. The data obtained in the presence of dADP are notable in that dADP is less effective at destabilizing the recA protein–ssDNA complex than the other nucleoside diphosphates. Also, the dADP–recA protein complex appears to induce a greater increase in the fluorescence of the etheno M13 DNA than the other nucleoside diphosphates. In fact, the relative fluorescence increase is similar to that observed in the high-affinity state.

Table II: Effect of Nucleotide Diphosphate on the ssDNA Binding Affinity of RecA Protein

	ST_{mp}[a] (mM NaCl)		RFI	
NDP	5 mM	9 mM	5 mM	9 mM
dADP	170	165	2.2	2.1
dCDP	110	100	1.5	1.4
CDP	110	100	1.5	1.7
ADP	110	85	1.4	1.3
TDP	85	80	1.6	1.6
GDP	100	75	1.4	1.3
UDP	90	60	1.7	1.6
dGDP	65	40	1.4	1.3

[a] Salt titrations were done with 5.4 μM etheno M13 DNA and 1.57 μM recA protein as described under Materials and Methods at 37 °C in 4 mM $MgCl_2$. Concentrations of 5 and 9 mM NDP were used to ensure saturation.

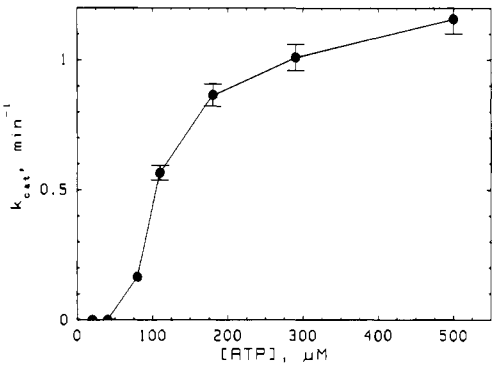

FIGURE 1: Effect of ATP concentration on the ATP hydrolysis rate in the presence of 10 mM calcium chloride. The rate of hydrolysis was determined as described under Materials and Methods. The concentrations of etheno M13 DNA and recA protein were 5.4 and 1.57 μM, respectively.

Binding of RecA Protein to ssDNA in the Presence of Calcium Chloride. Weinstock et al. (1981) reported that the hydrolysis of ATP is inhibited when $MgCl_2$ is replaced by $CaCl_2$. We have further characterized the mechanism of $CaCl_2$ inhibition in an attempt to study the effect of ATP binding to recA protein in the absence of hydrolysis. In the presence of $CaCl_2$, the observed rate of ATP hydrolysis is cooperative with respect to ATP concentration and yields an apparent K_m of 108 μM (Figure 1). This value is within a factor of 2 of that observed in the presence of $MgCl_2$. However, the maximal rate of hydrolysis (k_{cat}) is only 4% of that observed in the presence of $MgCl_2$. Thus, the data suggest that Ca^{2+} interferes with the actual hydrolytic step more than the binding of nucleotide.

Further evidence that nucleotide cofactor is bound to recA protein in the presence of $CaCl_2$ can be obtained from DNA binding data. The data in Table III show that, in the presence of $CaCl_2$, the affinity of recA protein for ssDNA is affected by nucleotide cofactors in a manner similar to that observed in the presence of $MgCl_2$. In both $MgCl_2$ and $CaCl_2$, the affinity of recA protein for DNA increases in the presence of ATP and decreases in the presence of ADP. However, the

Table III: Effect of $CaCl_2$ on RecA Protein ssDNA Binding Affinity

[divalent ion]	cofactor	ST_{mp}[a] (mM NaCl)		RFI[b]	
		Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
4	NC	275	275	1.9	1.9
10	NC	275	285	1.9	1.9
4	200 μM ADP	145	180	1.9	1.9
10	200 μM ADP	160	185	1.9	1.9
4	500 μM ATP[c]	600	490	2.3	2.3
10	500 μM ATP	600	515	2.3	2.3

[a] All experiments were done using 5.4 μM etheno M13 DNA and 1.57 μM recA protein in standard buffer conditions as described under Materials and Methods at 37 °C. Salt titration midpoints were determined as described under Materials and Methods. [b] Relative fluorescence increases were determined as described under Materials and Methods. [c] An ATP regenerating system was added to all experiments done using ATP, in the presence of $MgCl_2$.

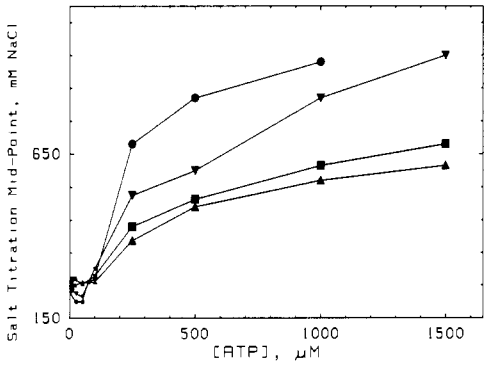

FIGURE 2: Effect of nucleotide concentration on the affinity of recA protein for ssDNA. Experiments were done at standard buffer conditions as described under Materials and Methods. The concentrations of etheno M13 DNA and recA protein were 5.4 and 1.57 μM, respectively. (●) ATPγS in 4 mM $CaCl_2$; (▼) ATP in 10 mM $MgCl_2$; (▲) ATP in 10 mM $CaCl_2$; (■) ATP in 4 mM $CaCl_2$.

affinity changes seem to be attenuated to a degree. Thus, the cofactor must be bound to the recA protein–ssDNA complex in order to show these nucleotide-specific effects on DNA binding affinity.

The relative fluorescence increase for each of the recA protein–ssDNA complexes is also shown in Table III. The data show that the high RFI induced by ATP is found in the presence of both $MgCl_2$ and $CaCl_2$. Thus, many of the ATP-dependent properties of recA protein are similar in the presence of either $MgCl_2$ or $CaCl_2$; these include the K_m value for ATP during hydrolysis, the effect of ATP on ssDNA affinity, and the effect of ATP on the RFI of the recA protein–etheno M13 DNA complex. These data suggest that the ATP-bound high-affinity state of recA protein in the presence of $CaCl_2$ is similar to that in the presence of $MgCl_2$, except hydrolysis is reduced by 96%.

Nucleotide Dependence of ssDNA Binding Affinity. The effect of ATP concentration, in the presence of either $MgCl_2$ or $CaCl_2$, is shown in Figure 2. In the presence of $MgCl_2$, there is a slight decrease in the affinity below 100 μM ATP. However, as the ATP concentration is increased above 100 μM, the stability of the recA protein–ssDNA complex increases. The concentration of ATP required to induce half of the maximal increase in the salt titration midpoint in $MgCl_2$ is approximately 500 μM ATP. In the presence of $CaCl_2$, there is no decrease in affinity at low ATP concentrations. (At concentrations of ATP above 250 μM and in the presence of $CaCl_2$, less than 10 μM ATP is hydrolyzed by recA protein during the experiment; below this ATP concentration, significantly less hydrolysis occurs. Therefore, the concentration of ATP in each experiment remains essentially constant.) The

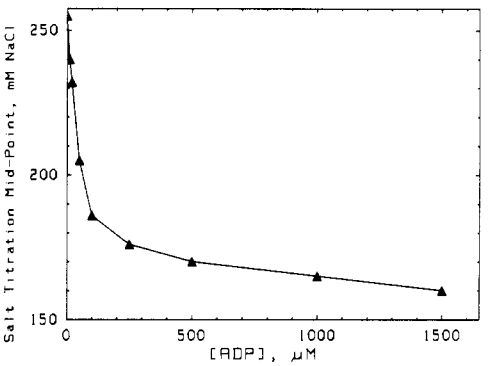

FIGURE 3: Effect of ADP concentration on the affinity of recA protein for ssDNA. Experiments were done at standard buffer conditions as described under Materials and Methods. The concentrations of etheno M13 DNA and recA protein were held constant at 5.4 and 1.57 μM, respectively, in 10 mM $CaCl_2$.

concentrations of ATP required to increase the salt titration midpoint to half of its maximum in the presence of 4 and 10 mM $CaCl_2$ are 350 and 275 μM ATP, respectively. Thus, the data suggest that recA protein has a higher affinity for ATP in the absence of hydrolysis than in the presence of hydrolysis.

The nonhydrolyzable ATP analogue ATPγS was shown to bind to recA protein with a higher affinity than ATP and to induce a high-affinity state in the presence of $MgCl_2$ that was not dissociable by NaCl (Menetski & Kowalczykowski, 1985). This analogue also induces a high-affinity state in the presence of $CaCl_2$, although the complex does dissociate at high NaCl concentrations. The dependence of the salt titration midpoint on ATPγS concentration is also shown in Figure 2. The concentration of ATPγS required to induce the half-maximal increase in the salt titration midpoint in the presence of $CaCl_2$ is 200 μM, which is less than that for ATP. Therefore, in $CaCl_2$ as in $MgCl_2$, recA protein has a higher apparent affinity for ATPγS than it does for ATP.

A comparison of the plateau values of the ATP and ATPγS concentration dependence curves in the presence of either $MgCl_2$ or $CaCl_2$ (Figure 2) shows that the presence of $CaCl_2$ reduces the apparent affinity of recA protein for ssDNA in the recA protein–ATP–ssDNA complex. The salt concentration required to disrupt the complex in the presence of $MgCl_2$ is greater than that required in the presence of $CaCl_2$. Thus, even though the major effect of Ca^{2+} on the recA–ATP complex is the inhibition of hydrolysis, it does, in addition, show an effect on recA–ssDNA affinity in the presence of cofactors. The data also show that ATPγS induces a higher affinity for ssDNA than ATP as measured by salt dissociation experiments. This is true in the presence of both $CaCl_2$ and $MgCl_2$ (Menetski & Kowalczykowski, 1985).

The affinity of recA protein for ssDNA has been shown to be dependent on the concentration of ADP (Menetski & Kowalczykowski, 1985). Increasing the ADP concentration, in the presence of magnesium chloride, decreases the affinity with an apparent K_d of 25 μM (Menetski & Kowalczykowski, 1985). In the presence of $CaCl_2$, the affinity of recA protein for ssDNA also decreases with increasing ADP concentration (Figure 3). The interaction of recA protein with Ca^{2+}–ADP can be characterized by an apparent K_d of 100 μM.

Effect of ATP/ADP Ratio on RecA Protein–ssDNA Binding Affinity. During the time course of ATP hydrolysis, the ATP concentration decreases while the ADP concentration increases. Thus, the molar ratio of ATP to ADP changes under these conditions. Since ATP hydrolysis is greatly reduced in the presence of $CaCl_2$, we were able to vary the ATP/ADP molar ratio to simulate hydrolysis and to determine

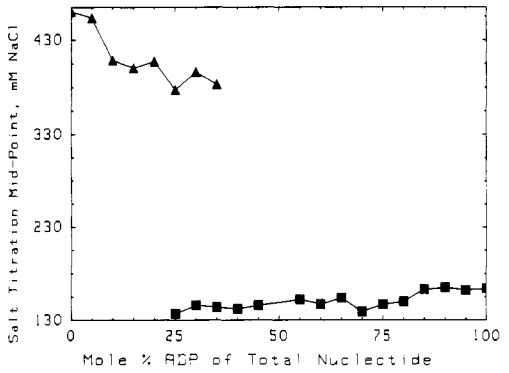

FIGURE 4: Effect of varying the ATP/ADP ratio on recA protein–ssDNA binding affinity. Experiments were done at standard buffer conditions as described under Materials and Methods at 37 °C with 10 mM $CaCl_2$. The concentrations of recA protein and etheno M13 DNA were 1.57 and 5.4 μM, respectively. Salt titration midpoints were determined as a function of the ATP/ADP molar ratio with the total nucleotide concentration held constant at 500 μM. The x axis represents the mole percent of ADP compared to the total concentration of nucleotide present.

Table IV: Effect of ATP/ADP Molar Ratio on the ssDNA Affinity of RecA Protein in the Presence of $CaCl_2$

[ATP] (mM)	[ADP] (mM)	% complex in each affinity state[a]	
		low	high
450	50	0	100
375	125	40	60
350	150	73	27
325	175	85	15
300	200	100	0

[a] Experiments were done at standard conditions as described under Materials and Methods at 37 °C and 10 mM $CaCl_2$. The concentrations of recA protein and etheno M13 DNA were 1.57 and 5.4 μM, respectively.

the affinity of recA protein for ssDNA at each ratio. Figure 4 shows the change in the salt titration midpoint with the mole percent of ADP. The concentration of total nucleotide was held constant, while the concentrations of ATP and ADP were varied reciprocally. At high ATP concentrations (a low mole percentage of ADP), the data show that all of the recA protein is in a high-affinity state; i.e., a high concentration of salt is required to disrupt the complex. At high ADP concentrations, all of the recA protein is in a low-affinity state. The transition between these two states is quite abrupt and occurs at ATP/ADP molar ratios of 2–3 or an ADP mole percent of 25–35 based on total nucleotide.

In the region of ATP/ADP molar ratios of 2–3 (25–35% ADP), the salt titration curves obtained are biphasic (Table IV). In this biphasic region, one fraction of the recA protein–DNA complexes has a low apparent ssDNA binding affinity and is disrupted at the salt concentration expected to disrupt the ADP–recA protein species. The other fraction has a high ssDNA binding affinity and is disrupted at high NaCl concentrations similar to that required for the ATP–recA protein species. As the ATP/ADP molar ratio decreases, the amount of protein in the high-affinity complex decreases, while the amount of protein in the low-affinity complex increases. Thus, in the presence of nucleotide cofactors, the data suggest that recA protein exists in either one of two different affinity states that is determined by the molar ratio of ATP to ADP.

Binding of RecA Protein to ssDNA Is Cooperative in the Presence of Calcium Chloride. Previous studies have shown that recA protein binds to ssDNA cooperatively [Menetski & Kowalczykowski, 1985; see Takahashi et al. (1986) for

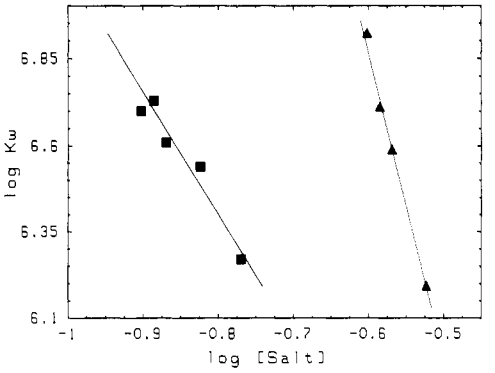

FIGURE 5: Effect of salt concentration on the affinity of recA protein for single-stranded DNA in the presence of $CaCl_2$. The units of $K\omega$ and salt concentration are M^{-1} and M, respectively. Protein titrations were conducted as described under Materials and Methods. The concentration of etheno M13 DNA was 1.57 μM. Experiments contained recA protein in 4 mM $CaCl_2$ at 37 °C using NaCl to vary the salt concentration: (▲) no cofactor ($y = -9.0x + 1.47$); (■) 500 μM ADP ($y = -3.3x + 3.76$).

effects of aggregation on cooperativity], with a monomer cooperativity parameter, ω, equal to 50. In the presence of $CaCl_2$, it should have been possible to determine whether this cooperative interaction of recA protein with ssDNA was similar in the presence of ATP. Unfortunately, in the presence of $CaCl_2$ (4 mM) and high NaCl concentrations (200 mM), the apparent association rate of the recA protein–Ca^{2+}–ATP complex is very slow and depends on the protein concentration. This has made the determination of K and ω in the presence of ATP and $CaCl_2$ impossible. However, protein titrations in the absence and presence of ADP have shown that the binding of recA protein to ssDNA in the presence of $CaCl_2$ is sigmoidal with respect to protein concentration (not shown, see Figure 5 for $K\omega$ values obtained). This sigmoidal behavior is indicative of cooperative binding and can be fit to an apparent monomer cooperativity parameter, ω, of 125 ± 50. This value of ω is slightly higher than that previously reported; however, we have found that the apparent monomer cooperativity, ω, increases slightly with temperature (unpublished observations) and these experiments were done at 37 °C. Thus, the binding of recA protein to ssDNA is also cooperative in the presence of $CaCl_2$.

Record et al. (1976) have shown that the number of ionic interactions between protein and nucleic acid can be determined from the slope of a plot of log K versus log [salt]. Previous studies have shown that the intrinsic affinity of recA protein for ssDNA is very dependent on salt concentration in the absence and presence of ADP ($\partial \log K/\partial \log$ [salt] = −11.1 and −4.7, respectively; Menetski & Kowalczykowski, 1985). As shown in Figure 5, the apparent affinity constants obtained from protein titrations in buffer containing $CaCl_2$ show a similar dependence in the absence and presence of ADP ($\partial \log K/\partial \log$ [salt] = −9 and −3.4, respectively). Thus, the apparent equilibrium binding properties of recA protein for ssDNA in the presence of Ca^{2+} are similar to those in Mg^{2+}.

Discussion

The DNA strand exchange reaction is known to require a nucleotide cofactor, ATP (McEntee et al., 1979). This cofactor has also been shown to increase the affinity of recA protein for ssDNA (Menetski & Kowalczykowski, 1985). However, little is known about the physical characteristics of the high-affinity state. In this paper, we report that the recA protein ssDNA binding affinity is increased by the presence of all nucleotide triphosphates that can be hydrolyzed by recA protein. However, nucleotide triphosphate hydrolysis is not

required to induce this high-affinity state. In the presence of $CaCl_2$, which reduces ATP hydrolysis to 4% of that observed in $MgCl_2$, the recA protein–ATP complex has a high affinity for ssDNA. Under these conditions of inhibited hydrolysis, we have also found that the affinity of recA protein for ssDNA is affected by both the concentration of ATP and the ATP/ADP molar ratio. Increasing the concentration of ATP in the presence of $CaCl_2$ increases the apparent affinity of recA protein for ssDNA in a saturable manner; decreasing the molar ratio of ATP to ADP concentration shifts the affinity abruptly from an ATP-like high-affinity state to an ADP-like low-affinity state.

During the analysis of the hydrolysis of nucleotides other than rATP, we have found that the major difference between nucleotides that are hydrolyzed (ATP, dATP, UTP, CTP, and dCTP) is their apparent binding affinity. The maximum rate of hydrolysis (V_{max}) of each of these nucleotides differs by at most 3.5-fold (see Table I). However, the apparent K_m values for each of these nucleotides vary more than 20-fold. Thus, the major difference in the hydrolysis properties of recA protein for these nucleotides is in the K_m. The dissociation constant for dATP binding to recA protein in the presence of ssDNA has been determined to be approximately 1.1 μM (Kowalczykowski, 1986). This value for the dissociation constant differs from the apparent K_m by about 50-fold. A similar discrepancy is observed for the binding and hydrolysis of ATP (Kowalczykowski, 1986). Furthermore, the binding of ATP and dATP to recA protein in the presence of ssDNA is hyperbolic (Kowalczykowski, 1986), while the rate dependence of ATP and dATP concentration is sigmoidal [ATP, Kowalczykowski (1986); ATP and dATP, this paper]. This suggests that binding of dATP or ATP is required but not sufficient to induce hydrolysis and may suggest some interaction between individual recA protein molecules that are bound to DNA.

All of the nucleotides that are hydrolyzed by recA protein increase the salt stability of the protein–DNA complex and, thus, the affinity of recA protein for ssDNA (see Table II). Previous studies have shown that recA protein has a higher affinity for etheno M13 DNA than for native ssDNA. Despite this difference, etheno M13 DNA behaves like unmodified ssDNA in that it supports all recA protein reactions that do not require homology (lexA proteolysis, ATPase activity, and coaggregation; unpublished observations). Thus, the hydrolyzable nucleoside triphosphates appear to affect the binding of recA protein to ssDNA in a manner similar to ATP. All nucleoside triphosphates that are not hydrolyzed, as well as all of the nucleoside diphosphates, reduce the affinity of recA protein for ssDNA. Since the affinity of the recA protein–etheno M13 DNA complex is influenced by these nucleotides, they must be bound to recA protein. Also, Kowalczykowski (1986) has shown that recA protein can bind to these nucleotides in the absence of DNA. Thus, in the case of the nucleoside triphosphates that are not hydrolyzed (i.e., GTP, dGTP, and TTP), the ability of recA protein to bind both nucleoside triphosphate and ssDNA is not sufficient to induce hydrolysis. These data suggest that the ability of recA protein to hydrolyze a nucleotide may be linked to the ability of that nucleotide to induce a specific conformation of recA protein, namely, the high ssDNA affinity state.

Another characteristic of the ATP-induced high-affinity ssDNA binding state is that ATP causes a greater increase in the fluorescence of the etheno M13 DNA–recA protein complex. The nucleotides that can be hydrolyzed by recA protein also induce a larger relative fluorescence increase (RFI) of the etheno M13 DNA relative to the no cofactor value. This change in the RFI is thought to be due to a conformational change of recA protein–etheno M13 DNA complex upon binding ATP (Menetski & Kowalczykowski, 1985). Therefore, the data show that these other nucleotides can induce a conformation change in the protein similar to that induced by ATP. However, the mechanistic reason that some nucleotides induce this high RFI while others do not is unknown.

In order to separate the effects of ATP binding and hydrolysis on the ssDNA binding affinity of recA protein, binding studies in the presence of $CaCl_2$ were carried out. The analysis of ATP hydrolysis in $CaCl_2$ shows that the dependence of rate on ATP concentration is cooperative with a K_m for ATP binding similar to that observed in $MgCl_2$ (108 and 60 μM in $CaCl_2$ and $MgCl_2$, respectively). However, the maximum rate of hydrolysis in $CaCl_2$ is only 4% of that observed in the presence of $MgCl_2$. Thus, the data show that ATP is bound by recA protein in the presence of $CaCl_2$ but catalysis is inhibited. These conditions have allowed the study of the ATP-induced high-affinity state of recA protein in nearly the absence of hydrolysis.

The apparent recA protein ssDNA binding affinity is affected by the concentration of ATP in the presence of either $MgCl_2$ or $CaCl_2$. As ATP concentration is increased, the apparent binding affinity increases in a saturable manner. The concentration of ATP that induces the half-maximal increase in apparent affinity is similar in the presence of $MgCl_2$ and $CaCl_2$ (500 and 350 μM, respectively). These data also suggest that the binding of ATP by recA protein in the presence of $CaCl_2$ is similar to that in $MgCl_2$. However, it is surprising that the concentration of ATP required to induce the half-maximal increase in affinity is almost an order of magnitude greater than that required for half-maximal ATPase activity in the presence of Mg^{2+}. Thus, these data suggest that hydrolysis is not required for the ATP-induced high-affinity state, because hydrolysis is negligible in the presence of $CaCl_2$; this is consistent with results obtained with the completely nonhydrolyzable analogue ATPγS. Also, since the nucleotide dependence of each process differs by an order of magnitude, the data suggest that ATP hydrolysis and the ATP binding induced high-affinity state are distinct, separable processes. However, the factors that induce high affinity may be those that stimulate hydrolysis.

The ssDNA-dependent ATP hydrolysis by recA protein has been shown to stop at a specific molar ratio of ATP to ADP (0.6 or 63% ADP of total; Cox et al., 1983). Also, Wu et al. (1982) have shown that in the strand exchange reaction, D-loop formation is more sensitive to ADP concentration than branch migration. Under conditions of reduced hydrolysis, we were able to directly assess the effect of the ATP/ADP molar ratio on the affinity of recA protein for ssDNA. We have found that the ssDNA binding affinity of recA protein changes abruptly at ATP/ADP molar ratios of 2–3. At this molar ratio, the affinity of recA protein for ssDNA switches from an ADP-like low-affinity state to an ATP-like high-affinity state. This change in affinity appears to be cooperative; that is, no intermediate affinity complexes are observed. It may be of significance that the high ssDNA affinity state of recA protein is more sensitive to inhibition by ADP than the ssDNA-dependent ATPase activity. If one step of the strand exchange reaction requires the high-affinity state while another requires only ATP hydrolysis, differential effects of ATP/ADP molar ratios would be expected. In light of what is already known about steps sensitive to the ATP/ADP molar ratio, it is attractive to suggest a hypothesis for the differential

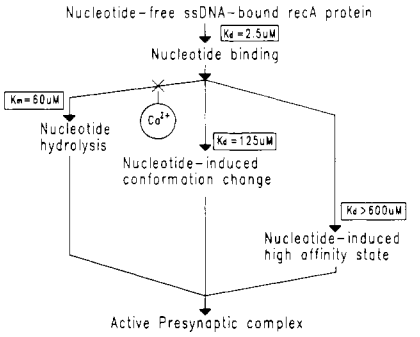

FIGURE 6: Mechanistic model for the effect of ATP upon binding to recA protein. ATP is bound to recA protein at low ATP concentrations. As the ATP concentration is increased, ATP hydrolysis is induced. At higher concentrations, ATP induces a conformational change in the protein, which is observed as an increase in the fluorescence of the recA protein–etheno M13 DNA complex. Finally, high concentrations of ATP induce a high ssDNA binding affinity. ATP can induce both the high ssDNA affinity state and the conformation change in the presence of $CaCl_2$, which severely reduces ATP hydrolysis. Thus, ATP hydrolysis is not absolutely required for induction of the conformation change and the high-affinity state.

ATP/ADP molar ratio sensitivity of D-loop formation and branch migration. It is possible that D-loop formation requires only the recA protein high-affinity complex, while branch migration requires only ATP hydrolysis. This hypothesis is consistent with the proposal that the ATP-bound high-affinity form of recA protein is important in dsDNA opening whereas dsDNA-dependent ATP hydrolysis (which occurs via a DNA intermediate with ssDNA character) is related to branch migration (Roman & Kowalczykowski, 1986; Kowalczykowski et al., 1987; Kowalczykowski, 1987). It has been shown that D-loop formation can occur in the absence of ATP hydrolysis when a nonhydrolyzable ATP analogue, ATPγS (which induces the ssDNA high-affinity state), is used (Riddles & Lehman, 1985; Honigberg et al., 1985). However, more experimentation is required to address this possibility completely.

The molecular explanation for this switch in affinity states is unknown; however, the data are also consistent with the active cluster hypothesis of Kowalczykowski (1986). This hypothesis suggests that the active ATP hydrolysis complex of recA protein is actually a specific number of protein molecules bound next to each other on the DNA and that each of the proteins in the cluster must be bound to ATP. If this hypothesis is correct and the ATP-induced high-affinity state is governed by similar interactions, then decreasing the ATP/ADP molar ratio may decrease the percentage of ATP in each functional cluster and induce the low-affinity state. Although this mechanism may offer an explanation for activation of recA protein ATPase activity and the high-affinity binding state, the specific molecular nature of the recA protein in these complexes is unknown.

The equilibrium and kinetic data described in this paper can be organized in a way that may give insight on the molecular requirements for the activation of recA protein in the strand exchange and repressor cleavage reactions. This scheme is presented in Figure 6 and represents a series of phenomenological events that occur upon increasing ATP (or NTP) concentration (these are not temporal events). Initially, at low ATP concentrations (<20 μM), recA protein–ssDNA complex binds nucleotide (K_d = 2.5 μM). This step, though required for ATPase activity, is not sufficient to induce ATP hydrolysis. Nucleotide hydrolysis is induced only when the nucleotide concentration is increased further (K_m = 60 μM). Nucleotide hydroysis is activated to its maximum at 75 μM ATP before the maximum induction of the relative fluorescence increase which occurs at $\approx$250 μM ATP. Induction of the higher RFI form of recA protein shows a hyperbolic dependence on ATP concentration with an apparent K_d of approximately 150 μM (Menetski & Kowalczykowski, 1987). Thus, a higher concentration of nucleotide is required to fully induce the recA protein conformation change than is required to fully induce ATPase activity. As ATP concentration is increased further, the induction of the recA protein high ssDNA binding affinity state occurs. A state with an affinity that is greater than the nucleotide-free complex begins to appear at approximately 100 μM ATP, but a significant increase is not evident until at least 250 μM. ATP hydrolysis on single-stranded M13 DNA has been shown to be inhibited by the *E. coli* ssDNA binding protein (SSB protein) below approximately 200 μM ATP (Kowalczykowski & Krupp, 1987). This corresponds to ATP concentrations that do not fully induce the high-RFI, high-affinity state of recA protein. Thus, this final state, which is ATPase active (in the presence of SSB protein), has a high relative fluorescence increase, and has a high affinity for ssDNA, is presumed to be required to maintain the SSB protein stimulated ATPase active state. Therefore, it seems likely that both high RFI and high affinity are required to form an active presynaptic complex under these conditions. This conclusion is consistent with the biochemical properties of recA430 (J. P. Menetski and S. C. Kowalczykowski, unpublished results) and recA142 (S. C. Kowalczykowski, Burk, and R. A. Krupp, unpublished results) which show defects in these properties and do not form active presynaptic complexes in the presence of SSB protein. However, though required properties, they are not necessarily sufficient for presynaptic complex formation in the presence of SSB protein, as evidenced by the inhibitory effect of SSB protein at 1 mM magnesium ion concentration.

In the presence of $CaCl_2$, we have been able to separate induction of the high-affinity complex from ATP hydrolysis; thus, hydrolysis is *not required* to induce the high relative fluorescence increase or the high-affinity state, but its nucleotide dependence places it before these later steps in this scheme. Resnick and Sussman (1982) have shown that the rate of λ repressor cleavage is similar in the presence of $CaCl_2$ and $MgCl_2$. Also, Phizicky (1983) has shown that repressor cleavage is stimulated by ATPγS. Therefore, it is apparent that only the high RFI conformation and/or the high-affinity state is required for repressor cleavage.

This model makes several predictions about the ability of recA protein to use alternative nucleotides in the strand exchange reaction. Our data suggest that all of the nucleotides that can be hydrolyzed by recA protein (i.e., dATP, UTP, CTP, and dCTP) could potentially stimulate the strand exchange reaction. However, the concentration of the nucleotide required to catalyze strand exchange may be as much as 10 times greater than its apparent K_m for NTPase activity. The nucleotide dATP can stimulate the strand exchange reaction at concentrations similar to those used in experiments with ATP (Weinstock & McEntee, 1981; J. P. Menetski and S. C. Kowalczykowski, unpublished observations). The nucleotide UTP was also reported to stimulate strand exchange to some extent (Weinstock & McEntee, 1981). It is possible that CTP and dCTP would also stimulate the strand exchange reaction; however, the concentration required may be as much as 10 mM NTP. This hypothesis is also consistent with the nucleotide requirements for λ repressor proteolysis; rGTP, dGTP, rCTP, dCTP, and TTP all fail to support the cleavage reaction at 1 mM NTP (Weinstock & McEntee, 1981). Thus, the nucleotide requirement of the strand exchange reaction may

be filled by any of the nucleotides that can be hydrolyzed by recA protein provided that a sufficiently high concentration of nucleotide is used.

Phizicky and Roberts (1981; Phizicky, 1983) have shown that dATP stimulates the proteolytic cleavage of λ repressor to a greater extent than ATP. Interestingly, the kinetic parameters for the hydrolysis of dATP show that recA protein binds and hydrolyzes this nucleotide more effectively than any other nucleotide including ATP. An explanation of these findings may arise from the effect of dADP on the binding of the recA protein–ssDNA complex. dADP reduces the apparent affinity of recA protein for ssDNA *less* than any other nucleoside diphosphate. Also, this nucleoside diphosphate appears to induce a relative fluorescence increase similar to that observed in the presence of ATP. However, the conformation of the recA–dADP complex cannot be the same as that of the recA–ATP complex because the affinity for ssDNA is lower in the presence of dADP. (Thus, it is not likely that the recA–dADP complex would be active in other recA protein activities such as lexA proteolysis.) Either the affinity difference or a different conformation induced by dADP may be responsible for an increase in processivity of dATP hydrolysis [see Menetski and Kowalczykowski (1987) for a discussion of processivity]. After hydrolysis, the dADP–recA protein complex should dissociate from the DNA less rapidly than the ADP complex, allowing more time for the exchange of dATP for dADP in the active site. This exchange may also be facilitated by the fact that the conformation of the recA protein–dADP complex is similar (though not the same) to that of the recA protein–NTP complex. Thus, recA protein may hydrolyze more dATP molecules per dissociation event than it would ATP molecules. This increase in processivity would result in the maintenance of the DNA–bound recA protein in a high-affinity state for longer periods of time. If this hypothesis is correct, then activities that require either high processivity or a long-lived high-affinity state would be stimulated. Preliminary studies on the dATPase activity of recA protein show that dATP is more effective than ATP at inducing the disruption of secondary structure and at the displacement of the *E. coli* ssDNA binding protein (SSB protein) from ssDNA (J. P. Menetski and S. C. Kowalczykowski, unpublished results).

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