recA Protein from *Escherichia coli*. A Very Rapid and Simple Purification Procedure: Binding of Adenosine 5′-Triphosphate and Adenosine 5′-Diphosphate by the Homogeneous Protein†

Susan M. Cotterill, Arnold C. Satterthwait,‡ and Alan R. Fersht*

ABSTRACT: The recA protein from *Escherichia coli* may be rapidly purified to homogeneity by a simple procedure involving only selective precipitation and one gel filtration step. The binding of ATP to the homogeneous protein has been measured by nonequilibrium dialysis. At pH 8.1 and 25 °C, the stoichiometry of the recA·ATP complex is 1:1 and the dissociation constant 24 μM. The binding of ADP to the enzyme and its complexes with single-stranded (ss) DNA and double-stranded (ds) DNA has been measured by equilibrium dialysis. In the absence of DNA, the binding is similar to that observed for ATP. The addition of ssDNA weakens the binding 3-fold. The addition of dsDNA causes a significant drop in the stoichiometry, suggesting an asymmetric distribution of active sites in the complex.

The recA protein of *Escherichia coli* is involved in a variety of biological processes. Genetic experiments have revealed that it is essential for general genetic recombination and for the postreplicative repair of DNA damage (Clark, 1973; Radding, 1978). It also has a central role in the "SOS" functions induced by DNA damage: induction of λ prophage, inhibition of cell division, mutagenesis, and inhibition of DNA degradation (Radman, 1975; Witkin, 1976). Furthermore, it stimulates its own synthesis on DNA damage (McEntee, 1977; Gudas & Mount, 1977; Little & Kleid, 1977; Emmerson & West, 1977). The cloning of the recA gene has led to the production of significant quantities of protein and to the intensive study of the relationship between its biochemical properties and its biological actions (McEntee & Epstein, 1977; Sancar & Rupp, 1979; Ogawa et al., 1979). The protein, of subunit molecular weight 37 800 (Horii et al., 1980; Sancar et al., 1980), has been found to be both a DNA-dependent ATPase [double stranded (ds) and single stranded (ss)] (Ogawa et al., 1979; Roberts et al., 1979; Weinstock et al., 1979; McEntee et al., 1979; Shibata et al., 1979a,b; West et al., 1980) and a single-stranded DNA-dependent and ATP-dependent protease (Roberts et al., 1979; Craig & Roberts, 1980). The latter activity is specific for an Ala–Gly bond in the lexA protein, the repressor for recA protein synthesis, and in the λ repressor (Markham et al., 1981; Horii et al., 1981). This could explain the induction of λ prophage and the derepression of the synthesis of recA protein on DNA damage since this may produce single-stranded DNA. The DNA-dependent ATPase activity is a consequence of the ATP-dependent annealing of single-stranded DNA to homologous double-stranded DNA.

Several purification procedures for the recA protein have now been published (Roberts et al., 1979; Weinstock et al., 1979; Emmerson et al., 1979; Cox et al., 1981; Shibata et al., Kuramitsu et al., 1981). These involve the steps of (I) cell lysis induced by lysozyme, EDTA, and detergent, (II) precipitation of the recA protein and DNA from the extract by polymin P and then elution of the recA protein with high salt, (III) ammonium sulfate precipitation, and (IV) column chromatography, e.g., hydroxylapatite followed by Sephacryl S-200 and DEAE-cellulose or phosphocellulose chromatography and ATP-induced elution from single-stranded DNA-cellulose. We present in detail a new purification procedure based on the observation that active recA protein precipitates quantitatively in nearly pure form from a solution of the ammonium sulfate fractions upon prolonged dialysis against 20 mM magnesium chloride. Further separation by gel chromatography gives essentially homogeneous recA protein free of the precipitating agent.

We report studies of the binding of ATP to the homogeneous protein under conditions where it is catalytically active by using the method of nonequilibrium dialysis (Colowick & Womack, 1969) and of the binding of ADP by using equilibrium dialysis.

Experimental Procedures

Materials. E. coli PE178 [N1462 [F⁻ (*lexA*⁻)] *spr51 sfiA″* Δ(*srl-recA*) 21 *thr*⁻ *pro*⁻ *ilvts*? *strA31 ara*⁻ *gal*⁻ *xyl*⁻ harboring pPE13 [=pBR322 (recA⁺)]] was a generous gift from Dr. P. T. Emmerson (Hickson et al., 1981). Radiochemicals were purchased from Amersham International (UK), 5′-ATP was from Boehringer, and all other biochemicals were from Sigma. Calf thymus DNA was denatured by boiling for 15 min at pH 8 (20 mM Tris-HCl and 0.1 mM EDTA)[1] and plunging into ice. Bacteriophage P22 double-stranded DNA was prepared as described by Weinstock et al. (1979).

Purification of the recA Protein. (*a*) *Cell Growth.* The enzyme was purified from PE178 grown in the following manner. Individual colonies were screened for high levels of recA protein production by picking from a plate (Luria plus 50 μg/mL ampicillin), growing with aeration in 100 mL of L broth plus 50 μg/mL ampicillin to A_{595} = 1, lysing the cells (as below), and examining the proteins by $NaDodSO_4$–polyacrylamide gel electrophoresis. Small stocks of log-phase cells that contained greater than 35% of their soluble protein as recA protein were stored at −80 °C after adding dimethyl sulfoxide to 7% and freezing in liquid nitrogen. These were used for inoculating larger cultures.

A kilogram of high yield cells (>35% recA protein) was obtained by the Imperial College Pilot Plant by inoculating 120 L of L broth plus 50 μg/mL ampicillin with 2 L of log-phase cells and growing overnight at 30 °C to A_{595} = 10. (The use of nalidixic acid does not significantly improve the yield and was not always used.) The cells were stored at −30 °C.

† From the Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, United Kingdom. *Received March 19, 1982.* This work was supported by SERC Grant GR/A94249.

‡ Present address: Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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It should be noted that the yield of recA protein is considerably reduced if inoculations are carried out with overgrown cells. It should be stressed that overgrowth of cells during serial growth leads to a loss of viable cells with a high recA protein content.

Two preparations of recA protein was described; volumes reported in parentheses refer to the first and second preparations, respectively. R buffer (Weinstock et al., 1979) is 20 mM Tris-HCl, pH 7.5 (20% base), 10% (v/v) glycerol, and 1 mM dithiothreitol (10 mM β-mercaptoethanol may be substituted for the dithiothreitol).

(*b*) *Cell Lysis.* The cell lysis procedure is essentially that of Weinstock et al. (1979). Frozen cells (50 g) were thawed in 50 mL Tris-HCl, pH 8.1 (50% base), and 25% sucrose (100 mL), frozen by dripping into liquid nitrogen, and thawed on ice overnight. All subsequent steps were carried out at 4 °C in a cold room. The thawed sucrose mixture was briefly blended to ensure that the cells were completely suspended. Lysozyme (100 mg) in 0.25 M Tris-HCl, pH 8.1 (50% base, 45 mL), was added and the mixture left for 15 min. A solution of 25 mM EDTA, pH 8.0 (45 mL), was added to the mixture and left for an additional 10 min. Finally, a solution of Brij 35 (1%), 50 mM Tris-HCl, pH 8.1 (50% base), and 1 mM dithiothreitol (170 mL) was blended into the cell mixture at low speed. Blending was continued for about 30 min until the viscosity was greatly reduced. A crude supernatant (fraction I) (290 mL, 300 mL) was collected following ultracentrifugation of the lysis mixture for 30 min at 35 000 rpm in a Beckman 45Ti rotor—a high-speed spin is essential for an effective separation of cell debris from the viscous supernatant.

(*c*) *Polymin P Precipitation.* The polymin P precipitation step is also taken from Weinstock et al. (1979). The crude supernatant (fraction I) was brought to 0.5% in polymin P (28 mL of 5.7% polymin P). The mixture was magnetically stirred vigorously to ensure the formation of a stringy precipitate; this consistency aids subsequent extractions. After 1 h, the precipitate was separated by ultracentrifugation (15 min at 25 000 rpm in the 45Ti rotor). The remaining pellet was broken up with a stirring rod in R buffer /0.5 M NaCl (100 mL, 150 mL) and homogenized in a Dounce homogenizer with a B pestle. The remaining precipitate was then immediately collected by centrifugation (15 min at 25 000 rpm). The supernatant, containing negligible recA activity, was discarded. The pellet was further homogenized in R buffer/1 M NaCl (50 mL, 75 mL) and left to soak for 1 h over ice. After centrifugation, a high-salt supernatant (48 mL, 72 mL) containing recA protein was obtained (fraction II). A third extraction of the pellet with R buffer/1 M NaCl did not elute any further significant amounts of recA protein and is not considered necessary.

(*d*) *Double Precipitation.* Fraction II was stirred with ammonium sulfate (0.28 g/mL) and left for 1 h on ice. After centrifugation (25 000 rpm/30 min), the precipitate (fraction III) was dissolved in R buffer/20 mM $MgCl_2$ (final volume 13 mL, 19 mL). The protein solution was dialyzed against 2 L of R buffer/20 mM $MgCl_2$. A light brown precipitate composed predominantly of recA protein formed slowly. [A reliable signal that precipitation has proceeded to completion is given when the precipitate has begun to collect at the bottom of the dialysis bag (complete settling will occur in a stationary dialysis bag; 15–25 h of dialysis is required).] The recA protein precipitate (fraction III) was collected by centrifugation at 25 000 rpm for 15 min and the dissolved in R buffer/1 M NaCl (22 mL, 15 mL) with the aid of the homogenizer to give fraction IV.

(*e*) *Gel Filtration.* Fraction IV was applied to either a Sephacryl S-200 (procedure 1) or a Sephacryl S-300 (procedure 2) column (2 L) and eluted with R buffer/1 M NaCl. In both cases, a significant proportion of protein eluted in the breakthrough volume. However, complete elution, because of overloading and/or size heterogeneity, required at least the first half of a column volume. This step provides further purification as well as separation from the factors responsible for precipitating recA protein at lower ionic strengths. Thus, after fractions are pooled, concentrated by Amicon filtration through a PM-10 filter, and dialyzed against R buffer, very little precipitation occurs. After removal of small amounts of precipitate (if any) by centrifugation, the concentrated fraction can be either subjected to other purification steps or dialyzed against R buffer/50% glycerol and stored at −20 °C (fraction IV).

(*f*) *Phosphocellulose Chromatography.* Although the recA protein is >98.5% pure after gel filtration and suitable for most purposes, further purification steps may be required. Previously (Cox et al., 1981), purification of recA protein on phosphocellulose has proved troublesome, as the enzyme is not fully retained by the resin, possibly due to contaminating polymin P. The fraction obtained from the gel filtration step was, however, reproducibly fractionated on phosphocellulose by using conditions previously described (Weinstock et al., 1979) to give fraction V. Thus 200–300 mg of recA protein can be purified on a 100-mL phosphocellulose column.

Phospholipid Composition of Precipitated Fractions. Phospholipids were extracted from precipitate II as follows. Approximately 100–200 mg of wet precipitate were homogenized in 2 mL of ethanol, centrifuged to remove rubbery debris, and concentrated to 0.2 mL by rotary evaporation. [Chloroform/methanol (2:1) extracted a similar mixture of phospholipids.] Phospholipids in the extract were identified by cochromatography with standards and color tests. A standard of dipalmitoyl-L-α-phosphatidyl-DL-glycerol was prepared by sonication of 3 mg in 1 mL of ethanol at room temperature. A sample of *E. coli* L-α-phosphatidylethanolamine in chloroform/methanol (9:1) was obtained from Sigma. Chromatography was carried out on silica gel H plates (Gurr & James, 1971) employing chloroform/methanol/acetic acid/water (85:15:10:4) as the eluting solvent. The major component of the phospholipid extract from precipitate II cochromatographed with phosphatidylglycerol and responded positively to the periodate–Schiff base reagent (Skipski & Barclay, 1969); it is therefore most likely some form of phosphatidylglycerol. A small amount of phosphatidylethanolamine (<10%) was also present and responded positively to ninhydrin spray.

Analysis for phosphorus (Ames, 1966) and protein (Bradford, 1976) in precipitate II dissolved in 1 M NaCl showed approximately 1 mol of phosphorus/mol of recA protein.

Routine Activity Assay. The DNA-dependent ATPase assay as described by Weinstock et al. (1979) was employed for determining activities. Reactions (50 μL) were carried out in 20 mM Tris-HCl, pH 7.5 (26% base), 10 mM $MgCl_2$, 1 mM dithiothreitol, 30 mM sodium chloride, 1 mM ATP (pH 7–8), and 5% glycerol with, or without, 500 μM single-stranded calf thymus DNA at 30 °C for 20 min. Approximately 1.5 μM recA protein was present in each reaction mixture. Reactions were carried out in 1.5-mL capped plastic tubes (we have noticed that some makes of "Eppendorf-type" tubes give lower activities than others; those obtained from Hughes & Hughes are suitable). The reactions were terminated by cooling on ice and the products separated by chromatography

Table I: Purification of recA Protein from *E. coli* PE178

fraction	total protein[a] (g)	total recA protein[b] (g)	recA protein (%)	recovery of act. (%)	total ATPase act. (units)[c]	ssDNA-dependent ATPase act. (units)[c]	sp act.[d]
			Procedure I				
(I) extract	8.4	2.8	33				
(II) polymin P	0.74	0.56	76	100	165	129	0.17
(III) ammonium sulfate	0.50	0.40	80	84	124	108	0.22
(IV) $MgCl_2$-induced precipitation	0.39	0.34	87	81	105	104	0.27
(V) Sephacryl S-200	0.286	0.254	89	49	63.6	63	0.22
(VI) phosphocellulose	0.17	0.17	>98	33	42.4	42	0.25
			Procedure 2				
(I) extract	8.5	3.0	35				
(II) polymin P	1.2	0.92	80	100	450	279	0.23
(III) ammonium sulfate	0.66	0.59	90	57	210	160	0.24
(IV) $MgCl_2$-induced precipitation	0.465	0.44	94	50	140	139	0.30
(V) Sephacryl S-300	0.243	0.240	>98.5	19	52.5	52	0.21

[a] Determined by staining with Coomassie Blue. [b] Determined from total protein and quantitative scanning of $NaDodSO_4$–polyacrylamide gels stained with Coomassie Blue. [c] 1 unit = 1 μmol of ADP produced per min at 30 °C (see text). [d] Units of ssDNA-dependent ATPase activity per milligram of protein.

on PEI sheets. Rates of hydrolysis were constant for 20 min under these conditions, and generally only a 20-min point was taken. Rate determinations were performed in triplicate. A unit of activity is defined as that amount of enzyme that hydrolyzes 1 μmol of ATP/min under the above conditions.

The concentration of homogeneous recA protein was determined spectrophotometrically by using $E_{280}^{1\%} = 5.17$ [calculated from the amino acid composition (Sancar et al., 1980; Horii et al., 1980) and the method of Mulvey et al. (1974); Kuramitsu et al. (1981) have calculated a similar value]. During the purification steps, the concentrations were determined by the Coomassie Blue method of Bradford (1976) by using purified recA protein as standard. Protein fractions examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (10%) Laemmli & Favre, 1973) were quantitatively analyzed after being stained with Coomassie Brilliant Blue G-250 by a Beckmann DU8 spectrophotometer equipped with a gel scanner and integrator. Integrations of absorbance values were checked by cutting out the peaks and weighing the paper.

Kinetic and Binding Experiments. The experiments were performed at 25 °C in standard buffer: pH 8.1 [40 mM Tris-HCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, and either 10 mM β-mercaptoethanol or 0.1 mM dithiothreitol), pH 6.2 [20 mM sodium maleate (3.5 mM monosodium and 16.5 mM disodium salt) replacing the pH 8.1 Tris-HCl], or pH 7.8 (potassium phosphate, 40 mM). The stock solutions of ATP and ADP were adjusted to the pH values of the relevant buffers.

Binding of ATP. The binding of ATP to the protein in the presence and absence of single-stranded DNA was measured by the nonequilibrium dialysis procedure of Colowick & Womack (1969). The apparatus consists of two chambers separated by a dialysis membrane. One chamber contains protein and labeled ligand, and the other chamber is a flow cell through which buffer is pumped. The rate of transfer of labeled ligand to the flow cell is proportional to the concentration of unbound ligand. Each binding isotherm may be determined over a period of only 20 min by using this procedure. Buffer was pumped through the bottom chamber (300 μL volume, 0.8-cm^2 cross section) of the apparatus at 5 mL/min and collected in scintillation vials in a fraction collector at 0.3-min intervals. The upper chamber, of similar dimensions to the lower, contained a solution of recA protein in the same buffer plus dithiothreitol. The two chambers were separated by a Sartorius SM 11539 dialysis membrane. Both were thermostated at 25 °C. A solution of [γ-^{32}P]ATP (5 μM) was added to the upper solution and its rate of transfer to the lower chamber measured by the procedure described by Colowick & Womack (1969) except that the amount of [γ-^{32}P]ATP in each fraction was measured directly by Cerenkov counting. Successive aliquots of unlabeled ATP were added to a concentration of 110 μM and then a final wash with 10 mM. Ten fractions were collected for each addition. The binding curve was constructed from the rates of transfer. The displacement of the [γ-^{32}P]ATP by ADP was also measured. Each binding isotherm was performed in at least triplicate. Aliquots of solution at the beginning and end of each experiment were analyzed by charcoal adsorption to show that there is negligible (<5%) hydrolysis of ATP during the run.

Binding of ADP. This was determined by equilibrium dialysis using the procedure described by Jakes & Fersht (1975).

Results

(A) Purification of recA Protein. PE175 is an excellent strain of *E. coli* for the production of recA protein, this constituting up to 50% or so of the soluble protein in the cell extract. It is important, however, when the inoculum is prepared for a large fermentation, not to allow the cells to grow out of log phase since cells that delete the *recA* gene grow much faster than those that are *recA*$^+$. We obtain about 1 kg of cell paste from a 120-L fermentation that will yield some 5 g of homogeneous recA protein by our second purification procedure (Table I).

Fractions I–III were obtained by published procedures (Weinstock et al., 1979; Cox et al., 1981). The next step in recently published procedures is the dialysis against a buffer containing 200 mM NaCl prior to chromatography on phosphocellulose to remove residual polymin P (Cox et al., 1981). However, prolonged dialysis under these conditions leads to the formation of variable amounts of a precipitate which was identified as recA protein. Shibata et al. (1981) and Craig & Roberts (1981) report that on dialysis against 50 mM phosphate or 0.1 M NaCl at this stage there is the formation of a precipitate which is then discarded. Kuramitsu et al. (1981) add the mixture of precipitate and solution directly to

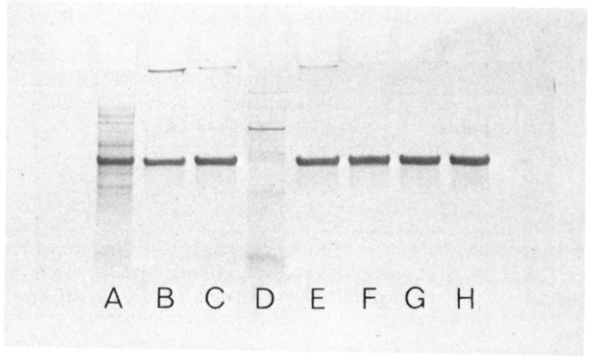

FIGURE 1: $NaDodSO_4$–polyacrylamide electrophoresis of recA protein fractions during purification. Track A, crude extract; track B, fraction II; track C, fraction III; track D, supernatant of fraction IV; track E, fraction IV, the $MgCl_2$-induced precipitate; track F, fraction V of procedure 1 (Sephacryl S-200 step); track G, fraction VI of procedure 1; track H, fraction V of procedure 2. The gels are heavily overloaded.

a phosphocellulose column. We find that the precipitate redissolves readily in a 100 mM phosphate (50% base, pH 6.8) or R-buffer containing 1 M NaCl; purified recA protein precipitated with polymin P also redissolves under these conditions and does not redissolve in 50 mM potassium phosphate. Since there is residual polymin P in this extract, some if not all of the precipitation is presumably due to its presence. On dialysis against R buffer alone, precipitation is variable, from 50 to 95% of the recA protein. However, when 20 mM $MgCl_2$ is present, precipitation of recA protein is nearly quantitative. Thus, under conditions where dialysis of fraction III gave 50% precipitation in the absence of $MgCl_2$, >95% precipitation was obtained in its presence. Precipitation also provides a significant purification step. At least 90% of the precipitated protein is recA protein (Table I). Furthermore, it has been separated from contaminating ATPase activity; 99% of this activity is DNA dependent. This figure is as high as any reported and the same as our most highly purified fractions. Phospholipids have also been identified as components of the precipitate. Initially, Sephacryl S-200 was used (procedure 1) followed by phosphocellulose chromatography. However, the use of Sephacryl S-300 is superior (procedure 2). In both cases, the recA protein emerges in the breakthrough volume, consistent with its being highly oligomerized under these conditions (Kuramitsu et al., 1981).

The stages of purification are illustrated in Figure 1. The gel has been heavily overloaded and overexposed to emphasize and detect minor bands of impurities. In track A is the crude extract (fraction I). In track B is fraction II, the high salt extract from the polymin P precipitate. The protein at the top of the gel is probably complexed with residual polymin P. There is less of this intractable protein after ammonium sulfate precipitation (track C, fraction III). The $MgCl_2$ precipitate (fraction IV, track E) does not appear on visible inspection of the gel to be any purer than fraction III. However, it is seen in Table I that there is a significant increase in the specific ATPase activity, and the fraction of the nonspecific ATPase activity that occurs in the absence of DNA drops from 15–20% to only 1% after the $MgCl_2$-induced precipitation. It is seen in track D of Figure 1 that the supernatant from the $MgCl_2$ precipitation contains very little recA protein and is enriched in the bands faintly visible in track C. Tracks F, G, and H are for the Sephacryl S-200 and phosphocellulose fractions of procedure 1 and S-300 fraction of procedure 2, respectively. No side bands can be seen on these with the naked eye. Quantitative scanning of tube gels with an absorbance of 2 units at the recA peak revealed no other absorbances of greater than 0.05 unit above the base line, hardly above noise level. The figures for the purities listed in Table I are the minimum values, assuming all side peaks are real and not due to noise or artifacts. These values may well be underestimates of the size homogeneity of the fractions.

Table II: Binding of ATP to the recA Protein[a]

pH	[recA] (μM)	stoichiometry (ATP monomer)	dissociation constant (μM)
8.1[b]	20	1.1	23
8.1	25	1.1	22
8.1	31	0.95	27
6.2[c]	20	0.7	28

[a] Determined by nonequilibrium dialysis at 25 °C, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 0.2 mM dithiothreitol plus indicated buffer. [ATP] varied from 5 to 110 μM. Several runs performed at each concentration. [b] 40 mM Tris-HCl. [c] 20 mM sodium maleate.

The activity of the homogeneous material compares well with that reported for other preparations. The turnover number for the ATPase activity is 8–10 min^{-1} compared with the values of 6.4–9 min^{-1} measured under similar conditions (Weinstock et al., 1981a,b). There is also a low content of endonuclease activity: less than 10^{-5} mol of supercoiled RFI DNA of ϕX174 is converted to the nicked RFII form min^{-1} (mol of recA protein)$^{-1}$ (unpublished data).

Phospholipids. When fraction IV was chromatographed in 1 M sodium chloride on Sephacryl S-200, it was noted that the fraction immediately following those containing recA protein had an absorbance at 280 nM that increased dramatically on warming to give sometimes visibly turbid solutions. This led us to test the $MgCl_2$-induced precipitate for phospholipids. Phosphatidylglycerol and phosphatidylethanolamine were identified as components. However, not more than 1 mol of phosphorus/mol of recA protein was present. Even if all the phosphate was from phospholipid, it would be surprising if it played a major role in precipitating the recA protein. We did find, however, that a commercial sample of *E. coli* phosphatidylethanolamine was effective in selectively precipitating recA protein from the mixture of proteins (lysozyme, pyruvate kinase, and bovine serum albumin) in the presence of $MgCl_2$.

(*B*) *Binding of ATP.* The hydrolysis of ATP catalyzed by the recA protein is sufficiently slow at both pH 8.1 (k_{cat} = 0.015 min^{-1}; K_M = 80 μM) and pH 6.2 (k_{cat} = 0.15 min^{-1}; K_M = 114 μM) at 30 °C (Weinstock et al., 1981c) that it is possible to measure the binding of the ATP to the enzyme by the nonequilibrium dialysis procedure of Colowick & Womack (1969). Over the period of the experiment (20 min), there is negligible hydrolysis as the concentration of ATP is raised from 5 to 110 μM in steps of 2 min. We find (Table II) that at 25 °C the apparent dissociation constant of the ATP·recA complex is 23–28 μM at both these values of pH with 20–30 μM recA protein. Binding appears to follow simple saturation curves as expected from the kinetics which were found to obey the Michaelis–Menten equation (Weinstock et al., 1981c). The stoichiometry of binding is 0.95–1.1 mol of ATP/mol of recA monomer at pH 8.1. There is a small, but consistently observed, drop in stoichiometry at pH 6.2 to 0.7.

(*C*) *Binding of ADP.* The binding isotherms, determined by equilibrium dialysis at 25 °C, are illustrated in Figure 2 and the data listed in Table III. At pH 8.1 and in the absence of DNA, the dissociation constant (31 μM) is similar to that found for the binding of ATP. The stoichiometry is 0.94, and there is no indication of a second binding site at up to 400 μM

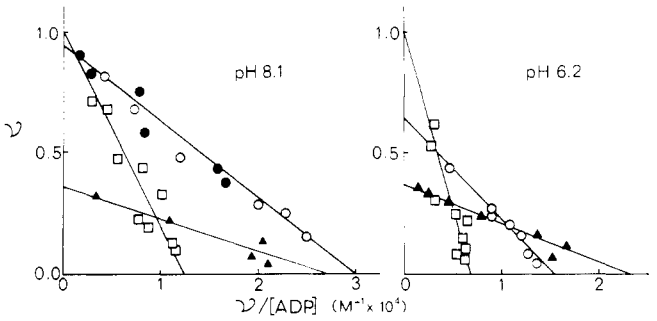

FIGURE 2: Scatchard plots of the binding of ADP to the recA protein at 25 °C at pH 8.1 and pH 6.2: (●) 80 μM recA protein and no DNA; (○) 40 μM recA protein and no DNA; (□) 40 μM recA protein and ssDNA (160 μM in nucleotide); (▲) 20 μM recA protein and 500 μM dsDNA at pH 8.1 or 40 μM recA protein and 200 μM dsDNA at pH 6.2.

Table III: Binding of ADP to the recA Protein[a]

pH	[recA] (μM)	DNA	[DNA]/[recA][e]	stoichiometry (ADP/monomer)	dissociation constant (μM)
8.1[b]	40, 80			0.94	31
8.1	73	ss	2	1.0	84
8.1	40	ss	4	1.0	77
8.1	73	ss	10	0.98	98
8.1	18	ds	5	~0.3	~14
8.1	40	ds	5	~0.5	~14
8.1	20	ds	25	~0.4	~14
6.2[c]	36			0.5	47
6.2	40			0.6	38
6.2	80	ss	2	1.1	140
6.2	40	ss	4	1.0	144
6.2	73	ss	4	1.0	109
6.2	36	ds	5	0.3	24
6.2	40	ds	5	0.35	24
6.2	40	ds	5	0.4	18
7.8[d]	40			0.42	110
7.8	40	ss	4	0.46	150
7.8	40	ds	5	0.33	100

[a] Determined by equilibrium dialysis at 25 °C, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 0.2 mM dithiothreitol plus indicated buffer. [ADP] varied from [recA]/5 to 10 [recA]. [b] 40 mM Tris-HCl. [c] 20 mM sodium maleate. [d] 40 mM potassium phosphate. [e] Nucleotides/monomer.

ADP. The addition of ssDNA increases the dissociation constant 3-fold while the stoichiometry is maintained at close to 1.0. The addition of dsDNA, however, causes the stoichiometry to drop to 0.3–0.5, the data for some reason, perhaps related to aggregation, being somewhat scattered. Low stoichiometry of binding to the recA·dsDNA complex is also observed at pH 6.2. There is also a low stoichiometry of binding in the absence of DNA at this pH, at about 0.5–0.6 mol of ADP/mol of recA protein, although the stoichiometry of 1.0 is found in the presence of ssDNA. The presence of ssDNA increases the dissociation constant 3-fold again. At pH 7.8 in the presence of 50 mM phosphate, the binding is weakened, and also low stoichiometries are observed.

(*D*) *Cross-Check of Binding of ADP by Nonequilibrium Dialysis.* The binding of ADP was determined by displacing bound [γ-^{32}P]ATP by using nonequilibrium dialysis. Modification of eq 3 of Fersht (1977) gives eq 1, where K_T is the

$$\frac{[ATP][E]_0}{[E{\cdot}ATP]} - [ATP] = K_T + [ADP]\frac{K_T}{K_D} \qquad (1)$$

dissociation constant for the ATP and K_D that for ADP, for the competitive inhibition of ATP binding by ADP. A plot of the left-hand side of eq 1 against ADP gives K_T and K_D. It is seen in Table IV that there is excellent agreement between K_D calculated this way and that found directly from equilibrium dialysis (Table III).

Table IV: Competitive Binding of ATP and ADP to the recA Protein[a]

[recA] (μM)	dissociation constant for ATP (μM)	dissociation constant for ADP (μM)
17	36	24
22	29	36
22	29	26

[a] Determined by nonequilibrium dialysis by the displacement of [γ-^{32}P]ATP by ADP using eq 1 at 25 °C, 10 mM $MgCl_2$, 10% (v/v) glycerol, 0.2 mM dithiothreitol, and 40 mM Tris-HCl at pH 8.1.

Competition between the binding of ATP and ADP has been observed in previous kinetic studies (Weinstock et al., 1981a).

Discussion

Purification Procedure. The precipitation of recA protein from a solution of the early ammonium sulfate precipitate has led us to invoke a new purification step which replaces ion-exchange chromatography. The overproducing strain of *E. coli*, PE178, used in this study has 35–50% of its soluble protein as recA protein and so requires only a 2- to 3-fold purification to homogeneity. The degree of purification obtained by the process of precipitation of the lipid–protein mixture by dialysis against low salt buffer containing $MgCl_2$ is even more impressive than indicated in Table I, however, when it is performed with extracts from poorly overproducing strains of *E. coli*. Repetitive rounds of precipitation against low salt and dissolution in high salt solutions are possible, and so repetition of this process allows purification to near homogeneity. Even after one round of precipitation, over 90% homogeneous protein may be obtained. As well as providing a useful rapid procedure for the large-scale purification of recA protein from overproducing strains, the $MgCl_2$-induced precipitation step should be even more useful for the isolation of recA protein and its mutants from other strains.

The precipitation of the recA protein from the crude ammonium sulfate cut is accompanied by the coprecipitation of a lipid fraction. This is perhaps not surprising since dialysis of lipids against $MgCl_2$ is a method of reconstituting lipid micelles. Since commercial samples of phosphatidylethanolamine were found to precipitate the enzyme and it has been reported (Gudas & Pardee, 1976) that the enzyme binds to the inner membrane of *E. coli* which is a rich source of this phospholipid, we investigated the possibility that phospholipids may have some biological role in stimulating the activity of recA protein. As phospholipids are also structurally reminiscent of DNA, we examined the possibility that phospholipids may be possible effectors of the recA ATPase activity. However, phospholipids extracted from their coprecipitates with the protein failed to stimulate its ATPase activity. It is possible that the association of recA protein with the lipids may be biologically relevant. But, as the recA protein is notoriously "sticky" and readily oligomerizes (Ogawa et al., 1979), it is likely that the association of the recA protein with the phospholipids is just a minifestation of nonspecific hydrophobic interactions.

Binding of ADP and ATP. The only quantitative studies so far on the binding of nucleotides to the recA protein have concerned ATP(γ)S, the nonhydrolyzable ATP analogue (Weinstock et al., 1981d; Craig & Roberts, 1981). Filter binding assays trap a complex of stoichiometry 1 mol of nu-

cleotide/mol of monomer in the presence of ssDNA, although no complex is observed in the absence of DNA. The present study shows that at pH 8.1 both ATP and ADP bind reasonably tightly to the free enzyme with stoichiometries close to 1.0 and dissocation constants about 30 μM (Tables II–IV). There appears to be just one ATP or ADP binding site per monomer. The presence of ssDNA weakens the binding of ADP some 3-fold. This implies that the dissociation constant for the complex of enzyme and ssDNA is also increased by a factor of 3 (calculated from a simple thermodynamic cycle). This is consistent with the observation of Weinstock et al. (1981b) that ADP increases the rate of dissociation of recA from its complex with ssDNA. It is probable that the release of recA from the complex with ssDNA and ATP involves the hydrolysis of ATP to ADP.

The binding of ADP to the complex of recA and dsDNA exhibits low stoichiometry at both pH 8.1 and pH 6.2. There is either a preexisting asymmetry or an ADP-induced asymmetry that prevents one-half to three-fourths of the binding being accessible to ADP. A lowered stoichiometry of binding is noted for ATP at pH 6.2. The enzyme is reported to undergo an ATP-induced oligomerization under these conditions to a filamentous complex (Weinstock et al., 1981b). The lowered stoichiometry could result from an asymmetry of the active sites in the complex.

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