Fonda, M. L. (1972) *Biochemistry 11*, 1304-1309.
Gani, D. (1985) *Annu. Rep. Prog. Chem., Sect. B 82*, 287-310.
Green, A. A., & Hughes, H. L. (1955) *Methods Enzymol. 1*, 67-70.
Hagino, H., & Nakayoma, K. (1968) *Agric. Biol. Chem. 32*, 727-733.
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) p 37, Wiley-Interscience, New York.
Kiick, D. M., & Cook, P. F. (1983) *Biochemistry 22*, 375-382.
Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497-525.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Martinez-Carrion, M., Cheng, S., Stankewicz, M. J., & Relimpio, A. (1975) in *Isoenzymes* (Markers, C. L., Ed.) Vol. II, p 567, Academic Press, New York.
Misono, H., Kawabata, Y., Toyosato, M., Yamamoto, T., & Soda, K. (1980) *Bull. Inst. Chem. Res. Kyoto Univ. 58*, 323-333.
O'Leary, M. H., Yamada, H., & Yapp, C. J. (1981) *Biochemistry 20*, 1476-1481.
Schowen, K. B. J. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 225, Plenum Press, New York.
Spector, T. (1978) *Anal. Biochem. 86*, 142-146.
Stevenson, D. E., Akhtar, M., & Gani, D. (1986) *Tetrahedron Lett. 27*, 5661-5664.
Stevenson, D. E., Akhtar, M., & Gani, D. (1990) *Biochemistry* (first paper of three in this issue).
Tobias, P. S., Cermosky, D., & Kallen, R. G. (1984) as cited in Kallen et al. (1985).
Velick, S. F., & Vavra, J. (1962) *J. Biol. Chem. 237*, 2109-2122.
Yamada, H., & O'Leary, M. H. (1977) *J. Am. Chem. Soc. 99*, 1660-1661.

Inhibition of RecA Protein Promoted ATP Hydrolysis. 1. ATPγS and ADP Are Antagonistic Inhibitors†

Jong Won Lee and Michael M. Cox*
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received December 26, 1989; Revised Manuscript Received May 8, 1990

ABSTRACT: ADP and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) inhibit recA protein promoted ATP hydrolysis by fundamentally different mechanisms. In both cases, at least two modes of inhibition are observed. For ADP, the first mode is competitive inhibition. The second mode is manifested by dissociation of recA protein from DNA. These are readily distinguished in a comparison of ATP hydrolyses that are activated by (a) DNA and (b) high (≈2 M) salt concentrations. Competitive inhibition with a significant degree of cooperativity is observed under both sets of conditions, although the DNA-dependent activity is more sensitive to ADP than the high-salt reaction. The reaction in the presence of poly(deoxythymidylic acid) or duplex DNA ceases when about 60% of the available ATP is hydrolyzed, reflecting an ADP-mediated dissociation of recA protein from the DNA that is governed by the ADP/ATP ratio. In contrast, ATP hydrolysis proceeds nearly to completion at high salt concentrations. At high concentrations of ATP and ATPγS, ATPγS also acts as a competitive inhibitor. At low concentrations of ATPγS and ATP, however, ATPγS activates ATP hydrolysis. These patterns are observed for recA-mediated ATP hydrolysis with either high salt concentrations or a poly(deoxythymidylic acid) [poly(dT)] cofactor, although the activation is observed at much lower ATP and ATPγS concentrations when poly(dT) is used. ATPγS can also relieve the inhibitory effect of ADP under some conditions. ATPγS and ADP are antagonistic inhibitors, reinforcing the idea that they stabilize different conformations of the protein and suggesting that these conformations are mutually exclusive. The ATPγS (ATP) conformation is active in ATP hydrolysis. The ADP conformation is inactive.

The recA protein of *Escherichia coli* promotes DNA strand exchange reactions in vitro that mimic key steps in homologous genetic recombination in vivo [for reviews, see Cox and Lehman (1987) and Radding (1988)]. The active species in this reaction is a nucleoprotein filament of recA protein containing one recA monomer for every three nucleotides or base pairs of DNA. The DNA in this filament is held in an extended (5.1 Å per base pair) and underwound (18 bp/turn) conformation in the presence of ATP or adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS).[1] RecA protein has a DNA-dependent ATPase activity producing ADP and P_i (Roberts et al., 1978; Weinstock et al., 1981a). ATP hydrolysis is required in this system for DNA strand exchange (Cox & Lehman, 1981). The precise molecular role of ATP hydrolysis is unclear. RecA

† This work was supported by National Institutes of Health Grant GM 32335.
* Author to whom correspondence should be addressed.

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, single-stranded binding protein; EDTA, ethylenediaminetetraacetate; DTT, 1,4-dithiothreitol; PEP, phosphoenolpyruvate; etheno-ssDNA, modified ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine; poly(dT), poly(deoxythymidylic acid); FI, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII, nicked circular form of the same DNA molecule; FIII, linear form of the same DNA molecule; FX, closed circular form of the same DNA molecule but underwound by approximately 40%; PEI, poly(ethylenimine); AMP-PNP, adenylyl β,γ-imidotriphosphate.

0006-2960/90/0429-7666$02.50/0

protein can also hydrolyze ATP without binding to DNA in the presence of very high (≈2 M) salt concentrations (Pugh & Cox, 1988a). The rates of the salt-activated reaction are similar to those of the DNA-dependent reaction at low salt concentrations.

ADP and ATPγS inhibit DNA-dependent ATP hydrolysis by recA protein (Weinstock et al., 1979, 1981b,c; Cox et al., 1983; Menetski et al., 1988). The mechanism of this inhibition is still under investigation. At least two factors appear to contribute to the inhibitory effect of ADP in this system. First, ADP acts as a competitive inhibitor of ATP hydrolysis. The effects of this inhibition on the kinetics of ATP hydrolysis are strongly influenced by cooperative interactions between recA monomers within the nucleoprotein filament (Weinstock et al., 1981b). Second, as binding and formation of the recA filament on DNA are required for ATP hydrolysis, inhibition can also result from a change of the DNA-binding properties of recA protein or the recA filament as a whole. The observed extent of recA protein promoted ATP hydrolysis corresponds only to about 60% of the input ATP for DNA-dependent ATP hydrolysis in the presence of SSB (Cox et al., 1983). These results can be explained by complete dissociation of recA protein when the ADP/ATP ratio exceeds 1.0 (Silver & Fersht, 1982; Cox et al., 1983; Soltis & Lehman, 1983; Menetski & Kowalczykowski, 1985; Chabbert et al., 1987; Menetski et al., 1988).

ATPγS is an ATP analogue that is hydrolyzed very slowly by recA protein. ATPγS also strongly inhibits DNA-dependent ATP hydrolysis by recA protein (Weinstock et al., 1979, 1981c; Craig & Roberts, 1980; Riddles & Lehman, 1985). RecA protein binds one ATPγS/monomer in the presence of ssDNA (Weinstock et al., 1981c).

RecA protein binds DNA very tightly in the presence of this analogue, and this stability has made recA–DNA–ATPγS complexes the primary focus of structural studies using electron microscopy (Flory et al., 1984; Chrysogelos et al., 1985; Stasiak & Egelman, 1986; Williams & Spengler, 1986). The mechanism of inhibition by ATPγS has not been examined in detail, but studies to date have revealed some interesting complexities. In a study using single-stranded calf thymus DNA as a cofactor, Weinstock et al. (1981b) provided evidence that ATPγS binds to recA protein tightly and acts as a simple competitive inhibitor. A second study by Silver and Fersht (1982), using the modified DNA cofactor εDNA, again found tight binding of ATPγS ($K_{app} \cong 0.5$ μM) but also noted a high degree of cooperativity in the binding of both DNA and ATPγS. The differences may reflect the tighter binding and longer filaments formed on εDNA by recA protein, as filament length on calf thymus DNA is limited by DNA secondary structure.

More recent studies show that ATPγS can also act as an activator under some conditions. While high levels of ATPγS virtually inactivate the protein, low levels of ATPγS prevent recA dissociation (Lindsley & Cox, 1989, 1990) with minimal effects on ATP hydrolysis and strand exchange. Menge and Bryant (1988) have also reported that hydrolysis of GTP by recA protein is activated by ATP and the ATP analogue AMP-PNP.

These effects potentially provide an avenue for the identification and characterization of recA protein conformational states and interactions between recA subunits within a filament. This information, in turn, is fundamental to an understanding of the role of ATP hydrolysis in the DNA strand exchange reaction. Evidence already exists for at least two conformations of recA protein from the work of Kowalczykowski and colleagues (Menetski & Kowalczykowski, 1985; Menetski et al., 1988). One induced by ATP binds to DNA with "high affinity", and a "low-affinity" state appears when ADP is present. These states may be related to the extended and collapsed states of the recA filament, respectively, observed in the electron microscope (Flory et al., 1984; Stasiak & Egelman, 1986; Williams & Spengler, 1986). Much evidence now exists that the states induced by ATP and ATPγS are roughly equivalent (Flory et al., 1984; Egelman & Stasiak, 1986; Stasiak & Egelman, 1988; Pugh et al., 1989).

In the present study, the effects of ADP and ATPγS are examined in more detail. Inhibition of DNA-independent ATP hydrolysis is investigated. ATPγS is shown to be an activator of ATP hydrolysis when both nucleotides are present at subsaturating concentrations. In addition, ATPγS and ADP are shown to be antagonistic inhibitors that clearly stabilize different conformations of recA protein.

Materials and Methods

Materials. *E. coli* recA protein was purified to homogeneity as previously described (Cox et al., 1981). The concentration of recA protein in stock solutions was determined by absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 0.59$ mg^{-1} mL cm^{-1} (Craig & Roberts, 1981). Bacteriophage T4 DNA ligase was purified by a published procedure (Davis et al., 1980). The concentration of the T4 DNA ligase stock solution was obtained by Bradford analysis (Bradford, 1976). One unit of T4 ligase is defined as the amount required to give 50% ligation of *Hin*dIII fragments of λ DNA in 30 min at 16 °C and a 5′ DNA termini concentration of 0.12 μM in a 20-μL assay mixture.

Poly(dT) was purchased from Sigma. The size of poly(dT) was measured by labeling the 5′ end of poly(dT) with ^{32}P according to a published procedure (Maniatis et al., 1982). The length of the poly(dT) was heterogeneous; over 95% of the polymers had lengths that ranged from 340 to about 1000 nucleotides, and the average length was about 650 nucleotides. The concentration of poly(dT) was determined by absorbance at 264 nm, using an extinction coefficient of $\epsilon_{264} = 8520$ M^{-1} cm^{-1}. The plasmid pBCS1, 6.5 kb, was described previously (Schutte & Cox, 1988). The concentration of dsDNA was determined at 260 nm, using an extinction coefficient of $\epsilon_{260} = 6580$ M^{-1} cm^{-1}. DNA concentrations are expressed as total nucleotides. The nicked circular duplex form (FII) of a plasmid was prepared by randomly nicking the negatively supercoiled circular DNA form (FI) with DNase I in the presence of ethidium bromide (Shibata et al., 1981). Greater than 95% of the DNA prepared in this way was present as nicked circular DNA as determined by scanning densitometry of photographic negatives from ethidium bromide stained agarose gels. The remainder was either linearized or circular and supercoiled.

ATP and ADP were purchased from Sigma, and ATPγS was purchased from Boehringer Mannheim. The purity of ATP, ADP, and ATPγS was checked by thin-layer chromatography (Shibata et al., 1981). Both ATP and ADP are at least 99% pure, and ATPγS was at least 90% pure. The major contaminant of ATPγS preparations was ADP. Concentrations of ATP, ADP, and ATPγS were determined at 259 nm, using $\epsilon_{259} = 15\,400$ M^{-1} cm^{-1}. Poly(ethylenimine) (PEI) cellulose paper was purchased from Brinkman.

Reaction Conditions. High-salt-activated recA protein ATPase activity was measured in 50 mM Tris acetate (80% cation), 17.5 mM magnesium acetate, 2% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT, and 1.4 M sodium acetate. The final pH after addition of all components was 8.0 at 25 °C.

DNA-dependent ATP hydrolysis was measured in a standard reaction buffer containing 25 mM Tris acetate (80% cation), 10 mM magnesium acetate, 5% glycerol, 0.1 mM EDTA, and 1 mM DTT. Final pH after addition of all components was 7.5 at 25 °C. Unless otherwise noted, the final concentration of recA protein was 5 μM, and the concentration of poly(dT), where this was used, was 10 μM. All reactions were done at 37 °C.

[^{3}H]ATP ATPase Assay. This assay was used to measure ATP hydrolysis in all experiments in which the effects of ADP are examined, and it is based on quantitating the conversion of [^{3}H]ATP to [^{3}H]ADP. ADP was separated from ATP by chromatography on PEI strips in 0.5 M formic acid and 1.0 M lithium chloride developing buffer as previously described (Weinstock et al., 1981a). For better resolution, 1.3 μL of a 20 mM mixture of unlabeled ATP and ADP was spotted on each lane to provide markers. For the high-salt-activated recA protein ATPase reaction, 1 μL of reaction solution was taken and diluted immediately with 5 μL of water to facilitate separation of ADP from ATP. Then 1 μL of diluted solution was spotted on PEI strips. For DNA-dependent ATP hydrolysis, 1 μL of reaction solution was taken and spotted directly on PEI strips. After the strips were developed, they were dried, and the ATP and ADP spots were visualized under a short-wave UV lamp and cut out. Radioactivity was quantified by liquid scintillation using a Beckman LS 3801 counter.

Spectrophotometric Assay for ATP Hydrolysis. In experiments where ADP was not present, a coupled spectrophotometric assay was employed (Pugh & Cox, 1988a). In brief, regeneration of ATP from ADP and phosphoenolpyruvate is coupled to the conversion of NADH to NAD$^+$, which can be monitored spectrophotometrically by a decrease in absorbance at 380 nm. Due to the high concentration of NADH used in this study, absorbances were measured at 380 nm, instead of 340 nm (its absorbance maximum), so as to remain in the linear region of the spectrophotometer. High concentrations of NADH were necessary to ensure that a steady state (end point) was reached under all conditions. No component of the coupling system limited the observed rate of ATP hydrolysis. ATP is constantly regenerated in this system so that no product accumulation occurs. All absorbance measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two six-position thermojacketed cuvette holders attached to a constant-temperature water circulator. The slit width was 2 nm. Reactions (0.4 mL) were contained in self-masking quartz cuvettes with a path length of 0.5 cm. Rates of ATP hydrolysis (μM/min) were calculated from $-\Delta A_{380}$ min^{-1} obtained at steady state, using an extinction coefficient of ϵ_{380} = 1210 M^{-1} cm^{-1} for NADH. As ATPγS contains ADP that can be converted to ATP, small changes in absorbance observed in the presence of ATPγS prior to the addition of recA protein were subtracted from all data.

DNA Underwinding Assay. This assay measures underwinding of the nicked duplex DNA substrate that results from binding of recA protein to the nicked duplex DNA as described previously (Pugh & Cox, 1987). When a nicked circular duplex DNA substrate is employed for binding, the recA protein induced underwinding of the DNA is topologically trapped by covalently sealing the nick with T4 DNA ligase. When recA protein is removed after sealing the nick with T4 DNA ligase, the underwinding is maintained in the form of supercoils. This highly supercoiled DNA substrate is resolved from the relaxed DNA substrate by agarose gel electrophoresis. Aliquots (20 μL) containing ATP, nicked duplex DNA (pBCS1 FII), and the other reaction components as appropriate were taken from a reaction mixture at indicated times and incubated with 2 μL of T4 DNA ligase (0.2 mg/mL final, $\simeq$2000 units) for 2 min at 37 °C. The reaction was stopped by the addition of gel loading buffer containing 1% sodium dodecyl sulfate, 12 mM EDTA, 0.05% bromophenol blue, and 5% glycerol. The samples were loaded on a 0.8% agarose gel and electrophoresed for 15 h at 40 mA in buffer containing 0.04 M Tris acetate (80% cation) and 1 mM EDTA with or without 1 μM ethidium bromide. When the gel was run in the presence of 1 μM ethidium bromide, running buffer was recirculated. Bands of DNA stained with ethidium bromide were illuminated with UV irradiation and photographed.

pH Shift Experiments. This is a protocol used for all experiments that used duplex DNA as a cofactor for ATP hydrolysis and as a binding substrate. As binding of recA protein to duplex DNA is slow at neutral pH, 20 μM recA protein was first incubated with 20 μM pBCS1 DNA (FII) at pH 5.9 to facilitate binding and then shifted by dilution 1:1 with a high-pH solution to make a final pH of 7.5 at 37 °C. The low-pH solution contained 20 mM sodium maleate (pH 5.9 at 25 °C), 10 mM magnesium acetate, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, and 3 mM ATP. Under these conditions, a steady-state binding of recA protein was achieved in the first 5 min. After incubation at 20 min at 37 °C, the pH was shifted to 7.5 at 37 °C by diluting 1:1 with a solution containing 50 mM Tris acetate (pH 9.2 at 25 °C), 10 mM magnesium acetate, 5% glycerol, and 1 mM DTT. An ATP regenerating system consisting of 18 units/mL pyruvate kinase, 2.9 mM phosphoenolpyruvate (PEP), and 0.44 mM KCl was used to keep ADP levels low prior to the pH shift. This amount of PEP is consumed after about 40 min of incubation under these conditions, and ADP starts to accumulate. The pH shift was carried out after 20 min of incubation, and measurable conversion of ATP to ADP is observed about 20 min after the shift. The dilution buffer contained 3 mM ATP so the total concentration of nucleotide (ATP and ADP) remains constant before and after the pH shift. DNA, recA protein, and ATP regenerating system components are diluted 2-fold. This protocol results in recA–dsDNA complexes at pH 7.5 in which all of the DNA is stably bound at the moment ADP begins to accumulate.

In the experiment in Figure 11, the initial reaction volume was 450 μL. Three aliquots were removed for the DNA underwinding assay prior to the pH shift. Of the remainder, 40 μL was removed just before the pH shift and diluted as described above into a high-pH solution containing ^{3}H-labeled ATP. Another 340-μL portion was subjected to the same pH shift without [^{3}H]ATP. ATP hydrolysis and DNA binding (DNA underwinding) were then followed in parallel for the remainder of the experiment using these two portions and the assays described above.

Results

ADP Inhibition of High Salt and Poly(dT)-Dependent ATP Hydrolyses by RecA Protein: Cooperativity with Respect to ATP. To characterize the inhibition of recA protein promoted ATP hydrolysis by ADP, standard double-reciprocal plots relating ATP hydrolysis to ATP concentration were used. The effects of ADP were examined for both high-salt-activated and poly(dT)-dependent ATP hydrolysis by recA protein. Steady-state rates (V_s) were taken from segments of these plots in which the rate of ATP hydrolysis was approximately linear for at least 5 min (not shown). V_s corresponds to the initial rate for the high-salt reactions and to the steady state established either immediately or after the short lag or burst

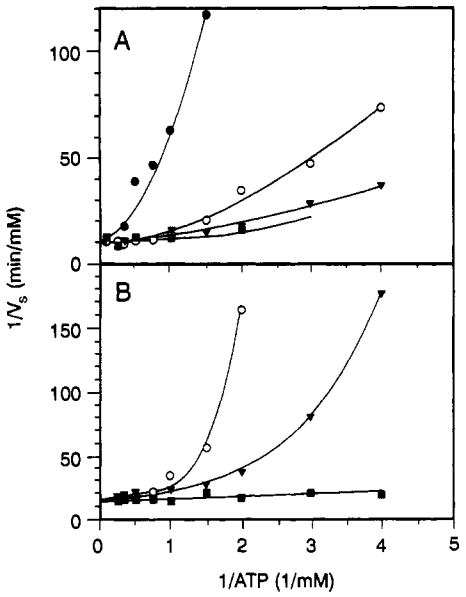


FIGURE 1: The effects of ADP on recA-mediated ATP hydrolysis: standard double-reciprocal plots. RecA protein and poly(dT) (where added) were 5 and 10 μM, respectively. Panel A: High-salt-activated ATP hydrolysis. ADP concentrations were 0 (■), 0.25 (▼), 0.5 (○), and 2 (●) mM. Panel B: Poly(dT)-dependent ATP hydrolysis by recA protein. ADP concentrations were 0 (■), 0.25 (▼), and 0.5 (○) mM ADP. For poly(dT)-dependent ATP hydrolysis at a high ADP concentration, recA protein was preincubated for 10 min with ADP before initiating the reaction with ATP.

[characterized in the following paper (Lee & Cox, 1990)] in the case of reactions with poly(dT) as the cofactor. The observed steady-state rate of ATP hydrolysis was independent of the order of addition of reaction components in all cases.

For both high-salt-activated and poly(dT)-dependent ATP hydrolyses by recA protein, ADP inhibits recA protein promoted ATP hydrolysis (panels A and B, respectively, of Figure 1). In both cases, the double-reciprocal plot is concave, indicating that homotropic positive cooperativity with respect to the substrate, ATP, exists in the presence of ADP. V_s approached the same maximal velocity, V_{max}, at high ATP concentrations irrespective of ADP concentrations for both the DNA-independent and poly(dT) reactions, indicating that ADP is a competitive inhibitor as reported previously for reactions with other cofactors (Weinstock et al., 1981b). Although the inhibition pattern is the same for both reactions, the rate of poly(dT)-dependent ATP hydrolysis by recA protein is much more sensitive to ADP than that of high-salt-activated ATP hydrolysis by recA protein at the same ATP concentration. The Hill coefficients for ATP in the presence of ADP for high-salt-activated and poly(dT)-dependent ATP hydrolyses are 1.8 and 2.5, respectively (not shown). The Hill coefficient for ATP in the poly(dT)-dependent reaction agrees well with the value of 3.3 obtained by Weinstock et al. (1981b) using ϕX174 ssDNA as a cofactor. A Hill coefficient of 1.8 for the high-salt-activated ATP hydrolysis indicates that there are at least two ATP binding sites per active unit. Pugh and Cox (1988a) also found that the active unit for high-salt-activated ATP hydrolysis by recA protein involves at least two to three monomers of recA protein. Apparent dissociation constants for ATP and ADP are given in Table I.

Cooperativity with Respect to ADP. To check whether there is a cooperative interaction between different ADP binding sites for high-salt-activated and for poly(dT)-dependent ATP hydrolyses by recA protein, the rate of ATP hydrolysis vs ADP concentration was plotted in Figure 2. In both cases, the curves are concave, indicating that there is

Table I: Apparent Dissociation Constants for ATP and ADP

reaction	$K_{D,app}$ (μM)[a] ATP	ADP
high salt	450	120
poly(dT)	200	120

[a] Apparent dissociation constants for ATP reflect the ATP concentration where the rate of ATP hydrolysis reaches half-maximum velocity. Apparent dissociation constants for ADP were obtained from the equation $K_{D,ADP} = [ADP]/[(ATP_{0.5,ADP}/ATP_{0.5}) - 1]$, where $ATP_{0.5} = K_{D,ATP}$ and $ATP_{0.5,ADP}$ is the ATP concentration where the rate of ATP hydrolysis reaches half the maximal velocity in the presence of the concentration of ADP in the numerator (Wong, 1975).

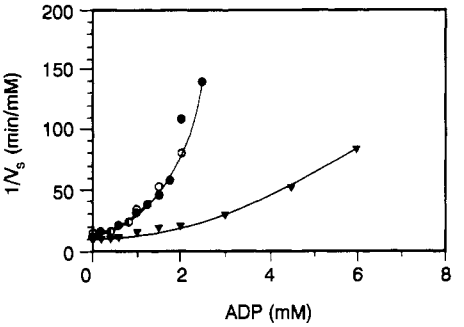


FIGURE 2: Effect of ADP on the rate of ATP hydrolysis at a fixed (2 mM) ATP concentration for high-salt-activated and for poly(dT)-dependent ATP hydrolysis. A Dixon-type plot was used to examine cooperativity with respect to ADP. Symbols denote salt-activated (▼) and poly(dT)-dependent ATP hydrolysis (●, ○). Open and closed circles denote results obtained with different orders of addition: (●) ATP and ADP added together or (○) recA preincubated with poly(dT) and ADP for 10 min before addition of ATP. Other reaction conditions are the same as described in the legend to Figure 1.

significant cooperativity with respect to ADP. Hill coefficients for ADP (data not shown) were 1.7 and 2.7 for high-salt-activated and for poly(dT)-dependent ATP hydrolyses by recA protein, respectively. The Hill coefficient observed in the presence of poly(dT) is again larger than that for the high-salt reaction. The Hill coefficients for ADP and ATP are very similar, consistent with the idea that ADP and ATP are binding to the same binding sites (Weinstock et al., 1981a,c).

Inhibition of RecA-Mediated ATP Hydrolysis by ATPγS. Inhibition patterns were again characterized with the aid of standard double-reciprocal plots relating substrate concentration and enzyme activity (Figure 3). Inhibition was examined both in the presence of high salt concentrations and with a poly(dT) cofactor.

The effects of ATPγS on DNA-independent (high-salt-activated) ATP hydrolysis are presented in Figure 3A. In the absence of ATPγS the line is curved, indicating a significant degree of cooperativity. In the presence of ATPγS (all concentrations tested) the lines are straight. The lines obtained at different ATPγS concentrations all exhibit a common intercept on the y axis, indicating that ATPγS is a reversible competitive inhibitor. The V_{max} for recA-mediated ATP hydrolysis under these conditions is 113 μM/min (k_{cat} = 22.6 min^{-1}). The apparent K_M for ATP is 450 μM (Table I), and the K_I for ATPγS is 100 μM. These results suggest that ATPγS binds more tightly to recA protein than ATP under these high-salt conditions.

The patterns observed with poly(dT) are significantly different (Figure 3B). The plots are concave, indicating a high degree of cooperativity for ATPγS concentrations above 5 μM. ATP hydrolysis is also much more sensitive to ATPγS. All lines again have a common intercept on the y axis, indicating competitive, reversible inhibition. The V_{max} and k_{cat} (82 μM

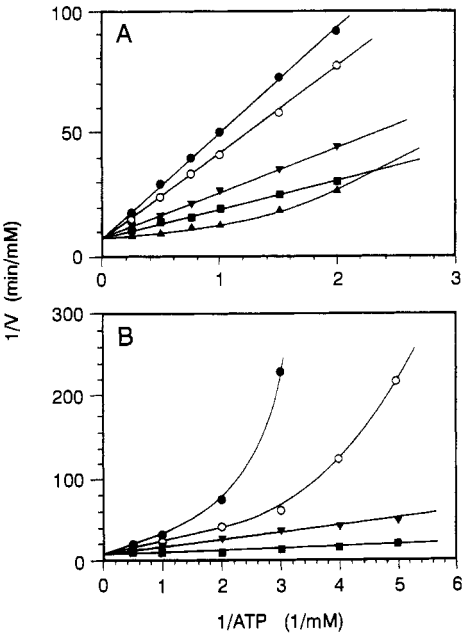


FIGURE 3: Effect of ATPγS on the rate of recA protein mediated ATP hydrolysis. The concentration of recA protein is 5 μM. Panel A: DNA-independent ATP hydrolysis in the presence of high salt (1.4 M sodium acetate). Concentrations of ATPγS (μM) were (▲) 0, (■) 200, (▼) 400, (○) 800, and (●) 1000. Panel B: Poly(dT)-dependent ATP hydrolysis. The concentration of poly(dT) was 10 μM. Concentrations of ATPγS (μM) were (■) 0, (▼) 5, (○) 7, and (●) 10. The spectrophotometric coupled assay for ATP hydrolysis described under Materials and Methods was used to obtain all data. ATPγS was added after preincubation of other reaction components at 37 °C for 10 min. After 10-min additional incubation, ATP was added to initiate reaction. In panel A data reflect steady-state rates observed about 1 min after addition of ATP. Data in panel B reflect steady-state rates observed between 10 and 20 min after initiation (see Figure 4).

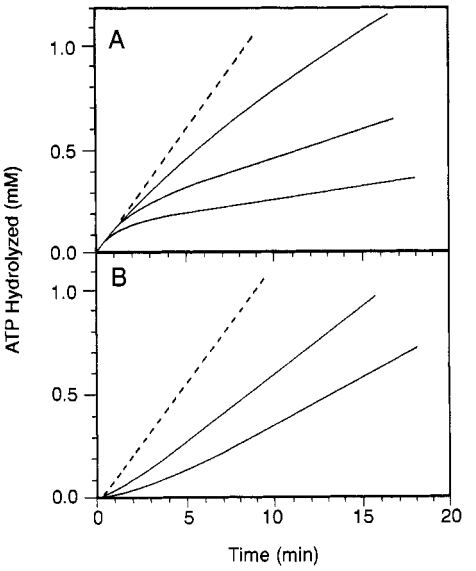


FIGURE 4: ATPγS inhibition of recA-mediated ATP hydrolysis: effect of order of addition of nucleotides. The poly(dT)-dependent reaction was monitored with the spectrophotometric coupled assay. Concentrations of recA protein, poly(dT), and ATP were 5 μM, 10 μM, and 2 mM, respectively. Dashed lines are reactions without ATPγS. Panel A: Slow onset of ATPγS inhibition in an ongoing reaction. RecA protein was preincubated with poly(dT) for 10 min, ATP was added, and incubation was continued for 10 min; then ATPγS was added at concentrations of 10, 25 or 50 μM in experiments indicated by solid lines, top to bottom. As plotted, $t = 0$ is the point at which ATPγS was added, and ATP hydrolyzed up to that point has been subtracted from the data. Note that ATP is continually recycled in this assay. Panel B: Lag in ATP hydrolysis when ATPγS is added prior to ATP. RecA protein and poly(dT) were preincubated for 10 min at 37 °C, ATPγS was added at either 5 or 10 μM (top and bottom solid lines, respectively), incubation was continued for 10 min, and then the reaction was initiated with ATP ($t = 0$ in panel B).

min^{-1} and 24.9 min^{-1}) are comparable to those measured in the high-salt conditions. The apparent K_M for ATP (200 μM) is somewhat lower than in high salt, but the K_I for ATPγS (1 μM) is much lower. ATPγS clearly has a much greater effect when poly(dT) rather than salt is used to stimulate ATP hydrolysis.

In the experiments from which Figure 3B was derived, burst kinetics were observed when ATPγS was added after ATP, and a lag was observed when ATPγS was added prior to ATP; i.e., the system exhibits hysteresis (Figure 4). The final steady-state rate of ATP hydrolysis is the same in either case, and the steady-state rates were used to generate Figure 3B. These transient kinetics have not been considered in more detail, but similar observations with ADP are characterized in the following paper.

ATPγS Activates RecA-Mediated ATP Hydrolysis at Low ATP and ATPγS Concentrations. The cooperativity observed in the DNA-independent reaction in high salt in the absence of ATPγS causes the bottom two lines in Figure 3A (at 0 and 200 μM ATPγS) to cross at low ATP concentrations. This suggests that, at sufficiently low ATP and ATPγS concentrations, ATPγS may actually stimulate rather than inhibit ATP hydrolysis. This prediction is borne out by the data in Figure 5. When ATP is present at or below its apparent K_M (at 200 and 500 μM), low levels of ATPγS stimulate the rate of ATP hydrolysis (Figure 5A). Optimal stimulation is observed at 80–100 μM ATPγS (approximately the observed K_I for ATPγS under these conditions), and net inhibition is observed at ATPγS concentrations above 300 μM. This activation is not observed at ATP concentrations significantly greater than the apparent K_M (e.g., at 1 mM ATP).

The activation also exhibited a strong dependence on recA protein concentration (Figure 5B). When 5 μM recA protein is used, the rate of ATP hydrolysis (at 200 μM ATP) is stimulated about 1.8-fold by 80 μM ATPγS. The stimulation is 2.8-fold when 20 μM recA protein is present.

Activation of ATP hydrolysis by ATPγS can also be observed in the poly(dT)-dependent reaction (Figure 5C). This is observed, however, at much lower ATP and ATPγS concentrations.

ADP and ATPγS Are Antagonistic Inhibitors. To a first approximation, both ADP and ATPγS appear to be simple competitive inhibitors, and under some conditions both exhibit the appropriate inhibition patterns. Both, however, exhibit another mechanistic component that serves to distinguish them. The activation of ATP hydrolysis by ATPγS is never observed with ADP, and the net dissociation of recA protein from DNA observed with ADP (Cox et al., 1983) is never observed with ATPγS. The mechanism of inhibition by these two nucleotides is therefore fundamentally different.

To examine this distinction in more detail, experiments were carried out on the DNA-independent reaction in high salt to examine inhibition patterns in the presence of both inhibitors. Theoretically, the combined effects could take any of three forms: (1) independent inhibition, i.e., the effects of the two inhibitors are simply additive; (2) synergism, where the effect of one would be enhanced by the other; and (3) antagonism, where the activity of one is reduced in the presence of the other.

In the first set of experiments, ATP hydrolysis was followed (without ATP regeneration) in the presence of varying concentrations of ATPγS (Figure 6). ATP was present at a concentration (1 mM) at which ATPγS activation is not ob-

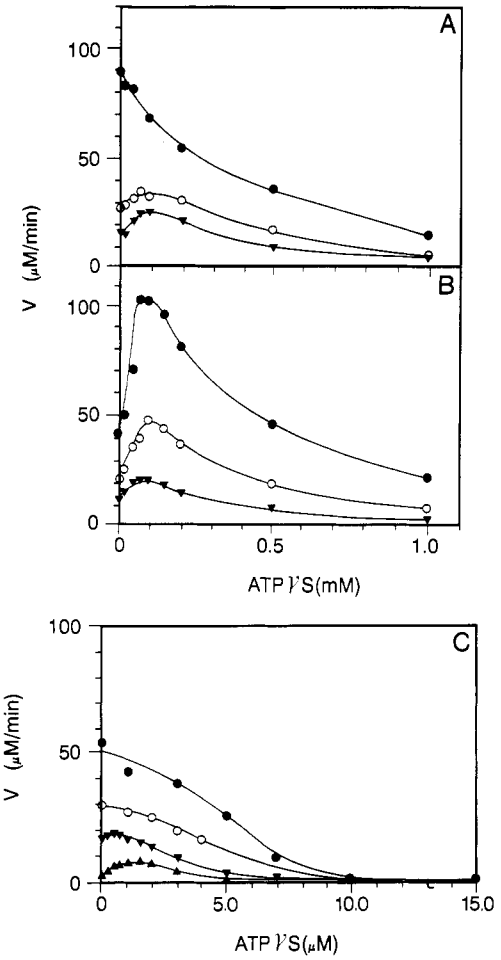


FIGURE 5: Activation of recA-mediated ATP hydrolysis by ATPγS. Data in panels A and B are from the DNA-independent reaction in the presence of 1.4 M sodium acetate. Panel A: Effect of ATP concentration. The recA protein concentration is 5 μM, and ATP concentrations are 1.0 mM (●), 0.5 mM (○), and 0.2 mM (▼). Panel B: Effect of recA protein concentration. ATP concentration is 0.2 mM. RecA protein concentrations were 20 μM (●), 10 μM (○), and 5 μM (▼). Panel C: Activation of recA-mediated ATP hydrolysis by ATPγS in the presence of poly(dT). The concentrations of recA protein and poly(dT) are 5 and 10 μM, respectively. ATP concentrations were 200 μM (●), 100 μM (○), 75 μM (▼), and 50 μM (▲).

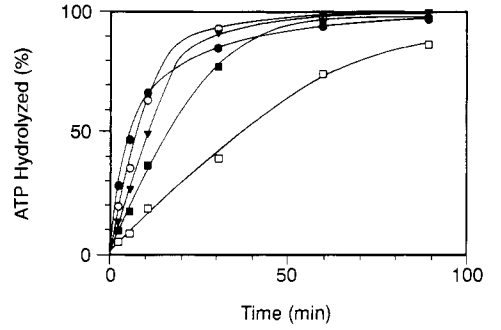


FIGURE 6: Effect of ATPγS on the course and extent of DNA-independent (high-salt) ATP hydrolysis by recA protein. The concentrations of recA protein and ATP were 5 μM and 1 mM, respectively. Reactions were initiated with a mixture of ATP and ATPγS after preincubation of other reaction components for 10 min at 37 °C. ATPγS concentrations were 0 (●), 50 μM (○), 100 μM (▼), 200 μM (■), and 500 μM (□).

served, and the initial rate of ATP hydrolysis is decreased at every ATPγS concentration examined. With the lower ATPγS concentrations, however, the rate of ATP hydrolysis surpassed the rate observed in the absence of ATPγS within 10–15 min. Without ATPγS, the rate of ATP hydrolysis slows with time as a result of substrate depletion, and also because

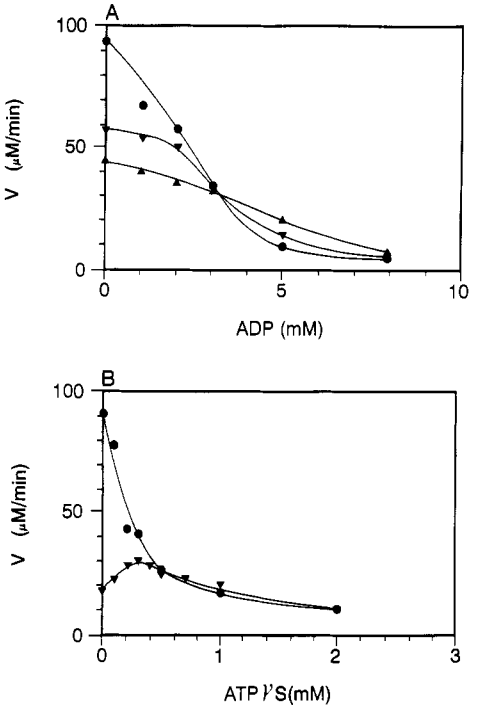


FIGURE 7: Combined effects of ATPγS and ADP on DNA-independent (high-salt) ATP hydrolysis by recA protein. The concentrations of recA protein and ATP were 5 μM and 2 mM, respectively. Reactions were initiated with a mixture of all nucleotides used in a given experiment after preincubation of other reaction components for 10 min at 37 °C. Panel A: Varying ADP concentration. Concentrations of ATPγS were 0 (●), 100 μM (▼), and 200 μM (▲). Panel B: Varying ATPγS concentration. ADP concentrations were 1.0 mM (●) and 5.0 mM (▼).

of the buildup of ADP, which acts as an inhibitor. Since substrate depletion must occur whether ATPγS is present or not, the long-term stimulatory effect of ATPγS can be explained only if it reduces the inhibitory effect of ADP; i.e., the two inhibitors are antagonistic.

This effect is examined more directly in Figure 7. First, initial rates of ATP hydrolysis are plotted as a function of ADP concentration for several fixed ATPγS concentrations (Figure 7A). ATPγS reduces the observed rate of ATP hydrolysis observed at low ADP concentrations, but *enhances* the rate observed at high ADP concentrations. This corresponds to a moderation of the slope of the ADP inhibition curve observed when ATPγS is present. In the experiments illustrated in Figure 7B, the ATPγS concentration is varied while the ADP concentration (1, 5 mM) is fixed. At 5 mM ADP, ATPγS exhibits a clear activation of ATP hydrolysis, with the optimal effect occurring at ~300 μM ATPγS. In both sets of experiments, ATPγS reduces the observed inhibitory effects of ADP.

Further Characterization of ADP-Mediated Dissociation of RecA Protein from DNA. The extent of ATP hydrolysis was determined under different reaction conditions and for different substrates (Figure 8A). In the high-salt reaction, recA protein hydrolyzes 100% of the added ATP, while in the low-salt solution with poly(dT) or nicked duplex DNA it hydrolyzes only about 60% of the added ATP, as observed previously (Cox et al., 1983; Menetski et al., 1988). The initial rates of the three reactions were similar, with measured k_{cat}s of 23 min^{-1}, 22 min^{-1}, and 19 min^{-1} for the high-salt, poly(dT)-dependent, and duplex DNA dependent reactions, respectively.

As indicated in the introduction, binding to duplex DNA is slow at pHs above 7.0. In order to follow ATP hydrolysis

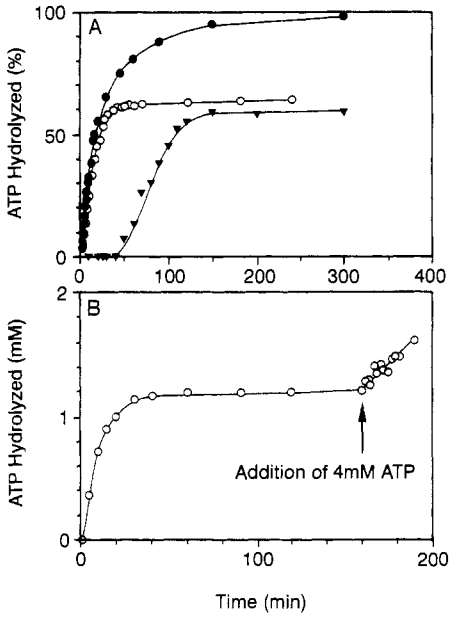
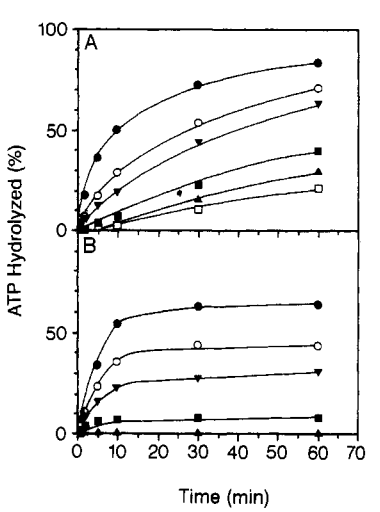


FIGURE 8: Extent of ATP hydrolysis for high-salt-activated, poly(dT)-dependent, and duplex DNA dependent ATP hydrolysis reactions. Panel A: Time courses of ATP hydrolysis for high-salt-activated (●), poly(dT)-dependent (○), and nicked duplex DNA dependent ATP hydrolyses (▼). For high-salt-activated ATP hydrolysis, 5 μM recA protein was preincubated at 37 °C for 10 min before adding 3 mM ATP. For poly(dT)-dependent ATP hydrolysis, recA protein (5 μM) and poly(dT) (10 μM) were preincubated in reaction buffer for 10 min before addition of ATP (2 mM final). For duplex DNA dependent ATP hydrolysis, a pH shift protocol was used with ATP regeneration to ensure complete binding prior to the onset of ADP accumulation as described under Materials and Methods. In the figure, $t = 0$ is the point at which the reaction ATP was added to initiate reaction. Steady-state (complete) binding was observed at pH 5.9 in less than 5 min. The pH was shifted from pH 5.9 to pH 7.5 at 20 min. The apparent lag reflects the time required to exhaust the ATP regenerating system (~40 min total—spanning the pH shift). The final concentrations of nicked pBCS1 (FII) DNA and recA protein were both 10 μM. Panel B: RecA protein (5 μM) was preincubated for 10 min at 37 °C with poly(dT) (10 μM). After 10 min, 2 mM ATP was added to initiate ATP hydrolysis by recA protein ($t = 0$). At t = 160 min (after ATP hydrolysis had reached an apparent end point), 4 mM more ATP was added (arrow). The amount of ATP hydrolyzed is represented in terms of concentration (mM) rather than in percentage (%) of total ATP so that both phases of the experiment can be plotted here.

mediated by intact nucleoprotein filaments on duplex DNA, binding was carried out at pH 5.9 in the presence of an ATP regenerating system and then shifted to pH 7.5 after 20 min (binding is complete in about 5 min under these conditions). No measurable ADP accumulates in this experiment until the PEP is exhausted (about 20 min after the pH shift—Figure 8A). This protocol is designed to ensure that binding is complete before ADP accumulation begins. The time course of ATP hydrolysis observed after the ATP regeneration system is exhausted is not affected by the pH shift itself or the amount of PEP added (at least up to 2.9 mM).

The cessation of ATP hydrolysis when only 60–70% of the ATP has reacted in the DNA-dependent reactions is not due to irreversible inactivation of recA protein. This is demonstrated by allowing a poly(dT)-dependent reaction to proceed to an apparent end point (~60% of the 2 mM ATP hydrolyzed), and then adding 4 mM additional ATP (Figure 8B). ATP hydrolysis begins immediately at a rate equivalent to that observed if 4.8 mM ATP and 1.2 mM ADP are added to initiate the reaction.

The extent of ATP hydrolysis does not change even if a mixture of ATP and ADP is added to initiate the reaction.



Initial ADP (%)	60 minutes ADP (%)
● 0.00	84.0
○ 33.3	80.0
▼ 50.0	82.0
■ 66.7	80.0
▲ 75.0	83.0
□ 83.3	87.0
● 0.00	63.6
○ 33.3	63.3
▼ 50.0	65.5
■ 66.7	70.0
▲ 75.0	75.0

FIGURE 9: Effect of the initial ADP/ATP ratio on the final extent of ATP hydrolysis for high-salt-activated and for poly(dT)-dependent ATP hydrolysis reactions. ATP hydrolysis starts by adding a mixture of ATP (1 mM final) and 0 (●), 0.5 (○), 1.0 (▼), 2.0 (■), 3.0 (▲), and 5.0 (□) mM final ADP concentration to preincubated (37 °C, 10 min) reaction mixtures containing recA protein (5 μM) in standard high-salt (panel A) or poly(dT) (panel B) reaction mixtures. The poly(dT) concentration in the panel B experiments was 10 μM. The plots reflect only the 1 mM labeled ATP present at $t = 0$, while the ADP percents listed at right reflect the total nucleotide concentration (ATP + ADP) present at $t = 0$ and at 60 min after reaction.

The effect of added ADP at $t = 0$ min on ATP hydrolysis is shown in Figure 9A,B. For the high-salt-activated reaction, ATP hydrolysis by recA protein still proceeds slowly after 60 min of reaction even if ADP and ATP are added in an 83:17 mixture initially (Figure 9A). However, for the poly(dT)-dependent reaction ATP hydrolysis stops when the ADP concentration becomes about 65% of the total ATP and ADP concentration regardless of the initial concentration of ATP and ADP (Figure 9B). Therefore, cessation of ATP hydrolysis when ADP constitutes more than 65% of the total is intrinsic to the DNA-dependent reactions. Even when the poly(dT) concentration was increased to 500 μM (30-fold excess relative to recA protein), no more than 70% of the available ATP was hydrolyzed (data not shown). As indicated in the introduction, this cessation of ATP hydrolysis reflects a dissociation of recA protein from DNA that is prominent whenever ADP and ATP concentrations are similar.

Small Amounts of ATPγS Prevent the ADP-Mediated Dissociation of RecA Protein from DNA. The antagonistic relationship between the effects of ATPγS and ADP is also evident in the DNA-dependent ATP hydrolysis reaction. It has been noted that low, subsaturating concentrations of ATPγS (1–10 μM) stabilize recA filaments bound to DNA with a minimal reduction in the rate of ATP hydrolysis (Lindsley & Cox, 1989). Since the cessation of DNA-dependent ATP hydrolysis at ADP/ATP ratios >1.0 is due to instability of the recA filament, addition of a small amount of ATPγS might increase the extent of ATP hydrolysis by stabilizing the filament. The extent of ATP hydrolysis in the DNA-dependent reaction in the presence of a small amount of ATPγS is shown in Figure 10. The initial rate of ATP hydrolysis is lowered when ATPγS is added. The extent of ATP hydrolysis for both the poly(dT)-dependent and nicked duplex DNA dependent ATP hydrolysis reactions, however, approaches 100% when the ATPγS is added (Figure 10B). ATPγS appears to prevent the ADP-mediated dissociation of recA protein from the DNA, allowing ATP hydrolysis to proceed further. The reaction progress curve is not hyperbolic

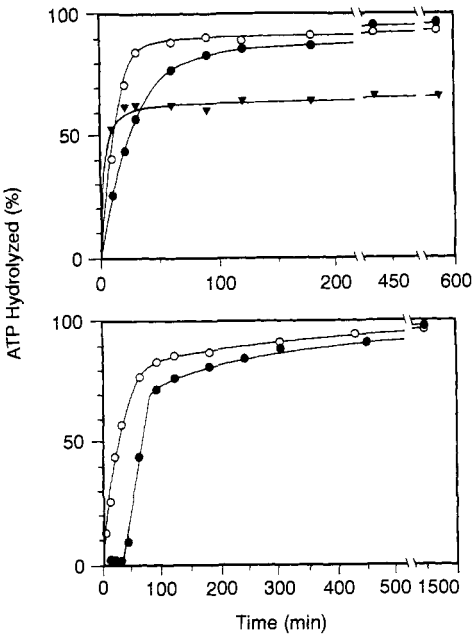


FIGURE 10: Effects of ATPγS on the course of poly(dT)- and DNA-dependent ATP hydrolysis by recA protein. Panel A: Poly(dT)-dependent ATP hydrolysis. RecA protein and poly(dT) concentrations were 5 and 10 μM, respectively. The ATP concentration was 1 mM. ATPγS concentrations were 0 (▼), 5 (○), and 10 μM (●). Reactions were initiated with ATP and ATPγS together after preincubation of other reaction components for 10 min at 37 °C. Panel B: Comparison of the poly(dT) (○) and duplex DNA (●) dependent reactions. For the poly(dT) reaction, poly(dT), recA, and ATP concentrations were 10 μM, 5 μM, and 1 mM, respectively. Reaction was initiated as in panel A. For the duplex DNA dependent reaction, a pH shift protocol and nicked circular pBCS1 DNA were used. Final concentrations of DNA, recA, ATP, and ATPγS were 10 μM, 10 μM, 1 mM, and 5 μM, respectively. The apparent lag in this reaction reflects the time required to exhaust a small amount of ATP regenerating system used in the pH shift protocol to ensure complete binding of recA to the DNA before ADP begins to accumulate.

but exhibits a rather abrupt change to a lower rate of ATP hydrolysis after 70–80% of the ATP is converted to ADP in the DNA-dependent reaction (Figure 10B).

To determine if these effects on the rate of ATP hydrolysis correlate with dissociation of the protein from DNA, DNA binding was examined directly with a ligation experiment (Figure 11). Binding of recA protein to duplex DNA extensively underwinds the DNA (Wu et al., 1983; Pugh & Cox, 1987, 1988b; Pugh et al., 1989), providing a convenient and unambiguous binding assay. The degree of binding of recA protein to nicked duplex DNA can be measured by trapping any recA protein induced topological changes in the DNA by ligation (Pugh & Cox, 1987). The extensively underwound form of DNA produced by ligating recA-bound duplex is referred to as form X and has been characterized in detail (Pugh & Cox, 1987, 1988b; Pugh et al., 1989). To facilitate binding to the duplex DNA at pH 7.5, a pH shift protocol was used (see Materials and Methods). An ATP regenerating system was also used, and the experiment was again designed so that the PEP was exhausted at about 35–40 min after the start of the reaction. To correlate binding of recA protein to nicked duplex DNA with the ADP concentration, the amount of form X produced and the extent of ATP hydrolysis were measured at the same time. To increase the resolution of topoisomers of the supercoiled duplex DNA produced in this experiment, gel electrophoresis was done in parallel in the absence (Figure 11A,C) and in the presence of 1 μM ethidium bromide (Figure 11B,D).

ADP-mediated dissociation was first examined in the absence of ATPγS (Figure 11A,B). In this experiment, binding of recA protein to nicked duplex DNA can be estimated from both the amount and the mobility of form X DNA. The amount of form X DNA reflects the fraction of duplex DNA molecules with some recA protein bound. The mobility of form X, which reflects the degree of underwinding, provides a measure of the amount of recA protein bound to each DNA molecule. In the time points taken before ADP begins to accumulate, virtually all of the ligatable DNA is found as the highly underwound form X, indicating that the DNA is bound up completely in recA nucleoprotein filaments (lane h). The DNA used in this experiment (pBCS1) is 6.5 kb and should have more than 2000 recA monomers bound to it at saturation. As the ADP concentration begins to increase, some dissociation of recA protein is observed. This is made evident at first by a change in the mobility of the underwound DNA (Figure 11A,B, lanes j–p). Initially, the changes are small (lanes j–l, 0–15% ADP). Almost all of the DNAs remain underwound to some extent (and therefore have some bound recA protein) until about 40% of the total nucleotide is present as ADP (lanes o–p). Beyond this point the fraction of DNAs with any underwinding decreases rapidly, approaching zero as the ADP levels off at about 60% of the total nucleotide (lanes r–t). A limited degree of cooperativity is evident in this dissociation process. This becomes clearer when 1 μM ethidium bromide is added to increase the resolution of topoisomers of supercoiled DNA during gel electrophoresis (Figure 11B). The DNA and recA complex is relatively homogeneous until about 10% of the ATP is converted to ADP (Figure 11B, lanes j and k, and unpublished results). With more ADP, the recA protein starts to dissociate, forming a population of DNA–recA protein complexes that are more heterogeneous (lanes l–p). Dissociation is not an all or none effect, however, and it is clearly characterized by a gradual reduction in the recA protein bound to each DNA rather than an abrupt dissociation of all the recA protein in individual complexes.

A similar experiment was done in the presence of a small amount of ATPγS (5 μM) (Figure 11C,D). Under these conditions the rate of ATP hydrolysis for poly(dT)-dependent ATP hydrolysis decreases by 30% compared to that observed without ATPγS. The ligation efficiency is also decreased somewhat in the presence of ATPγS. All of the ligatable DNA (≈60% of the total) is again present as form X before ADP begins to accumulate (lane h). In this case, conversion of ATP to ADP results in little detectable change in the nucleoprotein complexes until ADP represents more than 40% of the total nucleotide (Figure 11D, lane n). This indicates that the complexes are remaining essentially intact up to this point. As ADP increases between 45 and 60%, recA protein starts to dissociate appreciably (Figure 11C,D, lanes n and o). At 70% ADP (lane p), most of the recA protein has dissociated. The effect is not as complete as it is without ATPγS, however. At 80% ADP, some DNAs are still bound with a small amount of recA protein, which can hydrolyze ATP further (Figure 11C, lanes s and t), although most DNAs are unbound. This small amount of remaining bound recA protein explains the slow ATP hydrolysis observed at late times in Figure 10. ATPγS in small amounts stabilizes the filaments, an effect that is brought about by cooperative interactions between monomers within the filament. A high level of cooperativity is indicated by the relatively abrupt disintegration of the filaments as ADP increases from 45 to 70% of the total nucleotide.

Discussion

The principal conclusion is that ATPγS and ADP stabilize

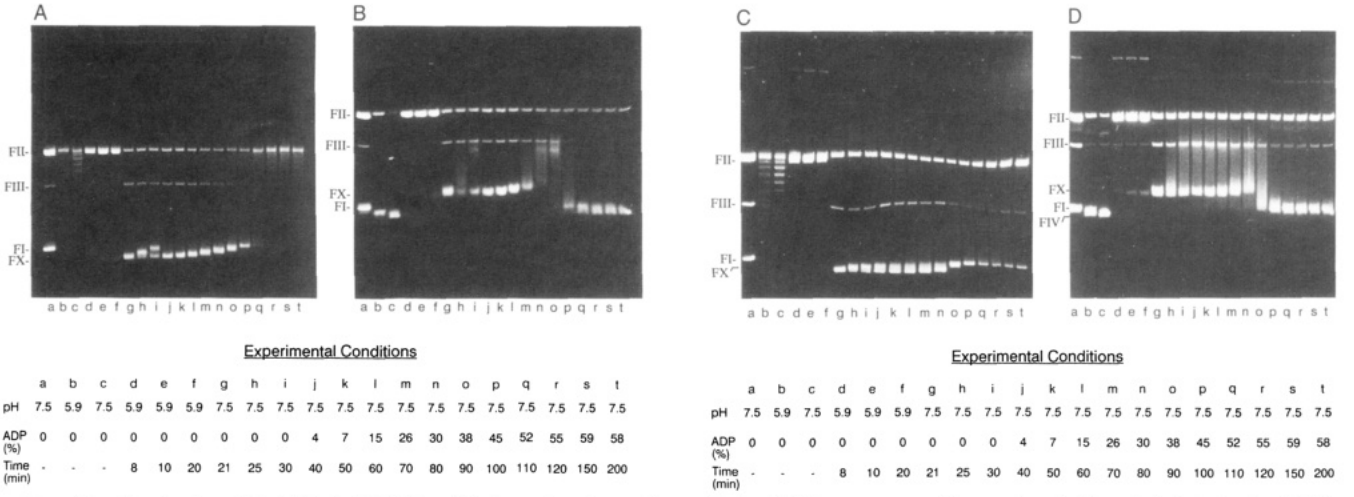


FIGURE 11: Panels A and B: Effect of ADP on binding of recA protein to duplex DNA as measured by underwinding of nicked duplex DNA. Experimental conditions are as described in the legend to Figure 8 except that the ATP concentration was maintained at 3 mM. The final reaction volume after the pH shift was 680 μL. At the indicated times, 20-μL aliquots were removed, ligated, and electrophoresed as described under Materials and Methods. Panels A and B are the same experiment electrophoresed in the absence or presence of 1 μM ethidium bromide, respectively. The extent of ATP hydrolysis was monitored by adding [^{3}H]ATP to a portion of the reaction mixture taken just prior to the pH shift. Lanes a–c in both panels are controls: (a) Markers for pBCS1 DNA in the supercoiled (FI), nicked (FII), and linear (FIII) forms; the FIII DNA band is a minor contaminant of FII DNA prepared as described under Materials and Methods. It is present in the experiment and is bound by recA, but is not ligated efficiently and has no effect on the result shown. (b) pBCS1 FII DNA ligated at pH 5.9 in the absence of recA protein. (c) pBCS1 FII DNA ligated at pH 7.5 in the absence of recA protein. Lanes d–t are from the experiment. The reaction was initiated at t = 0 min by adding ATP. RecA binding reaches a steady state at pH 5.9 in less than 5 min. Ligation in the presence of recA protein at this pH is very inefficient (lanes d–f). The pH shift occurs at t = 21 min, and the sample in lane g was taken immediately after the shift. The underwinding evident in the remaining lanes reflects recA protein binding as described in the text. The doublet of bands in lanes i and j appears to reflect a transient adjustment in the nucleoprotein filament after the pH shift. This has been observed in several experiments and is not reflected in any noticeable change in the rate of ATP hydrolysis [see Pugh and Cox (1987)]. The effect has not been characterized further. The binding state stabilizes about 15 min after the pH shift, and the onset of ADP accumulation (the ATP regeneration system is exhausted) occurs at about the same time. The band labeled "X" is FX DNA, the highly underwound species characteristic of complete binding by recA protein (Pugh et al., 1989). This band migrates ahead of FI DNA in panel A and behind FI DNA in panel B. Changes observed in lanes j–t reflect ADP effects. Rapidly migrating bands in panel B, lanes q–t, are relaxed, closed circular duplex DNAs (FIV). Panels C and D: ADP-mediated dissociation of recA protein from duplex DNA in the presence of ATPγS. The protocol for panels A and B was followed, except that the diluting buffer used for the pH shift in this case contained 10 μM ATPγS, giving a final concentration of 5 μM. At the indicated times, two 20-μL aliquots were removed, ligated, and electrophoresed as before. Panels C and D are again the same experiment electrophoresed in the absence or presence of 1 μM ethidium bromide, respectively. Lanes a–c in both panels are the same controls found in panels A and B. The reaction was initiated at pH 5.9 at t = 0 by addition of ATP. Ligation in the presence of recA protein at pH 5.9 was again very inefficient (lanes d–f), and material migrating with FII and FIII in all lanes is unligated. The pH shift occurs at t = 21 min, and ATPγS (5 μM) is introduced at this point. The underwinding observed in the ligated DNA from this point on reflects recA protein binding as described in the text. ADP accumulation begins between 30 and 40 min as the PEP in the ATP regeneration system is exhausted. Changes observed in lanes i–t reflect effects brought about by the increasing ADP concentration.

different conformations of recA protein. This is based primarily on the observation that in this system ATPγS and ADP are antagonistic inhibitors. There are two different effects of ADP: competitive inhibition and dissociation of the recA protein from DNA. ATPγS also has two different effects on the system. At high (saturating or nearly saturating) levels of ATP and ATPγS, ATPγS acts primarily as a simple competitive inhibitor. At low (subsaturating) concentrations of ATP and ATPγS, ATPγS *activates* recA-mediated ATP hydrolysis. ATPγS also stimulates the low levels of ATP hydrolysis observed when high ADP concentrations are present and greatly inhibits the ADP-mediated dissociation of recA protein from DNA. Neither ATPγS nor ADP is a simple competitive inhibitor. All of these results indicate that ATPγS and ADP stabilize mutually exclusive conformations of recA protein, both in the monomers to which they are bound and in neighboring monomers via protein–protein interactions.

Dissociation of recA from DNA is clearly mediated by the ADP/ATP ratio, becoming complete when the ratio approaches 1.5. These effects have been noted previously (Cox et al., 1983; Soltis & Lehman, 1983). The competitive inhibition and dissociative effects of ADP are extended here in several ways. First, dissociation of recA protein from duplex DNA as ADP accumulates is monitored by measuring the underwinding of duplex DNA associated with recA binding. Dissociation at 60% ADP was demonstrated for the DNA-dependent reactions in previous studies (Cox et al., 1983; Soltis & Lehman, 1983). The present study shows that this dissociation is gradual and roughly parallels the increase in ADP. The cessation of ATP hydrolysis when ADP is 60% of the total nucleotide corresponds to complete dissociation. Second, the DNA-dependent reaction is compared with DNA-independent ATP hydrolysis in high salt. To a first approximation, the high-salt reaction does not exhibit the ADP inhibitory effects associated with dissociation from DNA (e.g., the cessation of ATP hydrolysis when ADP/ATP = 1.5). The comparison provides an experimental means to partially separate the two effects. Third, the effects of cooperativity on the competitive inhibition patterns are explored. The high-salt- and DNA-dependent reactions exhibit Hill coefficients of 1.7–1.8 and 2.5–2.7, respectively, for both ATP and ADP. Together, the results suggest that both nucleotides bind to the same binding site but stabilize mutually exclusive states in a cooperative fashion.

It has previously been demonstrated that low levels of ADP actually enhance the initial rate of recA-mediated DNA strand exchange while inhibiting ATP hydrolysis (Cox et al., 1983; Rao et al., 1990). The results reported here indicate that the enhancement in strand exchange correlates with a modest reduction in recA protein binding. This reduction is shown here to take the form of a gradual reduction of the amount of recA in all filaments as ADP increases rather than a total

dissociation of some complete filaments. A reduction in the amount of recA protein bound to duplex DNA is detected at ADP/ATP ratios >0.05. The reason for the enhancement in strand exchange is unclear [optimum rates are observed at 30% ADP (Cox et al., 1983)]. The straightforward interpretation is that shorter filaments promote strand exchange more efficiently, although the enhancement could be related to some other molecular effect of ADP or a reduction of the torsional stress that may accompany strand exchange in this model system rather than size per se. Higher levels of ADP abolish both the strand exchange reaction and DNA binding.

The high-salt-activated ATP hydrolysis reaction is significantly less sensitive to ADP or ATPγS inhibition than the DNA-dependent process. Here, the effects of competitive inhibition and cooperativity can be observed in a system that, to a first approximation, lacks the complications due to dissociation of the DNA cofactor.

The antagonistic effects of ATPγS and ADP in the recA system mimic a phenomenon observed for several allosteric enzymes (Madsen & Shechosky, 1967; Monod et al., 1963). In these cases an antagonistic relationship is observed between a substrate or substrate analogue and an allosteric inhibitor. This opens the interesting possibility that the recA transitions may be mediated by nucleotide binding to a separate, regulatory binding site [as suggested previously by Menge and Bryant (1988)]. In this instance, however, the inhibitor is also the reaction product, and all results obtained to date can be explained adequately on the basis of binding exclusively at the active site.

That there are at least two states or conformations of recA protein is not a new insight by itself. Work in the laboratories of Kowalczykowski and Fersht (Silver & Fersht, 1982; Menetski & Kowalczykowski, 1985; Menetski et al., 1988) has distinguished high-affinity (for DNA) and low-affinity states of recA protein stabilized by ATP and ADP, respectively. These states were defined by changes in DNA binding observed when these nucleotides were added. In addition, these nucleotides cause gross structural changes in recA filaments, with ATP stabilizing an extended filament and ADP bringing about a "collapsed" state (Williams & Spengler, 1986). It is not clear, however, whether all of these studies are examining the same two conformations. Even if they are, it is not clear what relationship these two states have to the normal hydrolytic cycle. Finally, it does not follow that these two are the only conformations that are possible.

The simplest model one can draw is one proposed by Menetski and Kowalczykowski (1985), in which ATP hydrolysis is coupled to a DNA binding/dissociation cycle accompanied by interconversion of the two conformations. This model, however, does not incorporate the effects of cooperativity, and two sets of results indicate the system is considerably more complex. First, several studies have shown that there is no tight coupling between ATP hydrolysis and association/dissociation (Neuendorf & Cox, 1986; Lindsley & Cox, 1989). ATP is hydrolyzed uniformly throughout recA filaments (Brenner et al., 1987), and recA monomers in the filament interior generally do not dissociate as a result of ATP hydrolysis (Neuendorf & Cox, 1986; Shaner & Radding, 1987; Lindsley & Cox, 1989, 1990). The ADP-mediated dissociation of recA from DNA is mediated by the ADP/ATP ratio and requires a significant level of ADP that will not be present when ATP is regenerated. The second set of results involves the recA filament itself. ATP hydrolysis in the filament does not result in an accordion-like interconversion of the extended state observed in the presence of ATPγS and a collapsed state observed in the presence of ADP (Egelman & Stasiak, 1988; Pugh et al., 1989). More subtle conformation changes may occur during ATP hydrolysis (Egelman & Stasiak, 1988), but the state of the overall filament during ATP hydrolysis very closely resembles the state observed in the presence of ATPγS (Flory et al., 1984; Egelman & Stasiak, 1986, 1988; Pugh et al., 1989).

A more complete model that incorporates cooperativity can be summarized as follows. There are two conformations of recA, one (T) with a higher affinity for ATP than the other (D). The D conformation is stabilized by ADP. The T state corresponds to the high-affinity state of Kowalczykowski and colleagues, and the extended form of the filament (Flory et al., 1984; Chrysogelos et al., 1985; Menetski & Kowalczykowski, 1985; Stasiak & Egelman, 1986; Williams & Spengler, 1986; Menetski et al., 1988), while the D state corresponds to the low-affinity state of Menetski and Kowalczykowski (1985) and the collapsed form of the filament (Flory et al., 1984; Stasiak & Egelman, 1986, 1988). Binding of ATPγS to the active site of a recA monomer has two effects: it blocks the binding of ATP to that monomer (competitive inhibition) but stabilizes the T conformation in adjacent subunits through cooperative protein–protein interactions. The latter effect can result in activation of ATP hydrolysis when ATPγS and ATP are both subsaturating. Under these conditions there is not enough ATP alone to shift the equilibrium sufficiently toward the T state. Because it binds more tightly, ATPγS shifts the balance toward the T state at subsaturating concentrations. Likewise, binding of ADP has two effects: competitive inhibition and stabilization of the D conformation in adjacent subunits. The latter effect results in dissociation when the ADP/ATP ratio is high enough, possibly because the two states are structurally incompatible. This theme is developed further in the following paper (Lee & Cox, 1990).

The model described above still leaves open the question of what occurs during normal (nondissociative) ATP hydrolysis by monomers within a recA filament. The possibilities include (but are probably not limited to) the following: (1) ATP hydrolysis involves conversion of an individual monomer from the T state to the D state, but the presence of one or two adjacent monomers bound to ATP (T state) prevents dissociation and causes rapid recycling to the T state. (2) ATP hydrolysis results in no significant conformation changes, with the T state maintained by means of cooperative interactions with adjacent recA monomers. (3) ATP hydrolysis results in heretofore uncharacterized structural changes to conformational intermediates unrelated to the D state. For possibilities 2 and 3, the D state represents an inactive conformation that is inaccessible unless sufficient ADP is present so that significant contiguous tracts of recA monomers in a filament bind ADP.

Also unresolved is the relationship between the D state and dissociation of recA from DNA. In the following paper, evidence is presented that recA protein binds to DNA in the D state [as previously reported by Menetski and Kowalczykowski (1985) and Bryant et al. (1985)]. Dissociation may occur not as a result of conversion to the D state per se, but only when an incompatible mixture of T and D conformations is present.

Registry No. 5′-ATP, 56-65-5; 5′-ADP, 58-64-0; poly(dT), 25086-81-1; ATPγS, 35094-46-3.

References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brenner, S. L., Mitchell, R. S., Morrical, S. W., Neuendorf, S. K., Schutte, B. C., & Cox, M. M. (1987) *J. Biol. Chem. 262*, 4011–4016.

Bryant, F. R., Taylor, A. R., & Lehman, I. R. (1985) *J. Biol. Chem. 260*, 1196–1202.
Chabbert, M., Cazenave, C., & Helene, C. (1987) *Biochemistry 26*, 2218–2225.
Chrysogelos, S., Register, J. C., III, & Griffith, J. (1985) *J. Biol. Chem. 288*, 12624–12631.
Cox, M. M., & Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3433–3437.
Cox, M. M., & Lehman, I. R. (1987) *Annu. Rev. Biochem. 56*, 229–262.
Cox, M. M., McEntee, K., & Lehman, I. R. (1981) *J. Biol. Chem. 256*, 4676–4678.
Cox, M. M., Soltis, D. A., Lehman, I. R., DeBrosse, C., & Benkovic, S. J. (1983) *J. Biol. Chem. 258*, 2586–2592.
Craig, N. L., & Roberts, J. W. (1980) *Nature 283*, 26–30.
Craig, N. L., & Roberts, J. W. (1981) *J. Biol. Chem. 256*, 8039–8044.
Davis, R. W., Botstein, D., & Roth, J. R. (1980) in *Advanced Bacterial Genetics*, pp 116–119, 196, and 197, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Egelman, E. H., & Stasiak, A. (1986) *J. Mol. Biol. 191*, 677–697.
Egelman, E. H., & Stasiak, A. (1988) *J. Mol. Biol. 200*, 329–349.
Flory, J., Tsang, S. S., & Muniyappa, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7026–7030.
Lee, J. W., & Cox, M. M. (1990) *Biochemistry* (following paper in this issue).
Lindsley, J. E., & Cox, M. M. (1989) *J. Mol. Biol. 205*, 695–711.
Lindsley, J. E., & Cox, M. M. (1990) *J. Biol. Chem. 265*, 9043–9054.
Madsen, N. B., & Shechosky, S. (1967) *J. Biol. Chem. 242*, 3301–3307.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, pp 122–127, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Menetski, J. P., & Kowalczykowski, S. C. (1985) *J. Mol. Biol. 181*, 281–295.
Menetski, J. P., Varghese, A., & Kowalczykowski, S. C. (1988) *Biochemistry 27*, 1205–1212.
Menge, K. L., & Bryant, F. R. (1988) *Biochemistry 27*, 2635–2640.
Monod, J., Changeux, J. P., & Jacob, F. (1963) *J. Mol. Biol. 6*, 306–329.
Neuendorf, S. K., & Cox, M. M. (1986) *J. Biol. Chem. 261*, 8276–8282.
Pugh, B. F., & Cox, M. M. (1987) *J. Biol. Chem. 262*, 1326–1336.
Pugh, B. F., & Cox, M. M. (1988a) *J. Biol. Chem. 263*, 76–83.
Pugh, B. F., & Cox, M. M. (1988b) *J. Mol. Biol. 203*, 479–493.
Pugh, B. F., Schutte, B. C., & Cox, M. M. (1989) *J. Mol. Biol. 205*, 487–492.
Radding, C. M. (1988) in *Genetic Recombination* (Kucherlapati, R., & Smith, G., Eds.) pp 193–229, American Society for Microbiology, Washington, DC.
Rao, B. J., Jwang, B., & Radding, C. M. (1990) *J. Mol. Biol.* (in press).
Riddles, P. W., & Lehman, I. R. (1985) *J. Biol. Chem. 260*, 170–173.
Roberts, J. W., Roberts, C. W., Craig, N. L., & Phizicky, E. M. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 917–920.
Schutte, B. C., & Cox, M. M. (1988) *Biochemistry 27*, 7886–7894.
Shaner, S. L., & Radding, C. M. (1987) *J. Biol. Chem. 262*, 9211–9219.
Shibata, T., Cunningham, R. P., & Radding, C. M. (1981) *J. Biol. Chem. 256*, 7557–7564.
Silver, M. S., & Fersht, A. R. (1982) *Biochemistry 21*, 6066–6072.
Soltis, D. A., & Lehman, I. R. (1983) *J. Biol. Chem. 258*, 6073–6077.
Stasiak, A., & Egelman, E. H. (1986) *Biophys. J. 49*, 5–6.
Stasiak, A., & Egelman, E. H. (1988) in *Genetic Recombination* (Kucherlapati, R., & Smith, G., Eds.) pp 265–307, American Society for Microbiology, Washington, DC.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126–130.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981a) *J. Biol. Chem. 256*, 8829–8834.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981b) *J. Biol. Chem. 256*, 8845–8849.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981c) *J. Biol. Chem. 256*, 8850–8855.
Williams, R. C., & Spengler, S. J. (1986) *J. Mol. Biol. 187*, 109–118.
Wu, A. M., Bianchi, M., DasGupta, C., & Radding, C. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1256–1260.