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# Interaction of RecA Protein with a Photoaffinity Analogue of ATP, 8-Azido-ATP: Determination of Nucleotide Cofactor Binding Parameters and of the Relationship between ATP Binding and ATP Hydrolysis†

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ABSTRACT: The binding and cross-linking of the ATP photoaffinity analogue 8-azidoadenosine 5′-triphosphate (azido-ATP) with recA protein have been investigated, and through cross-linking inhibition studies, the binding of other nucleotide cofactors to recA protein has also been studied. The azido-ATP molecule was shown to be a good ATP analogue with regard to recA protein binding and enzymatic function by three criteria: first, the cross-linking follows a simple hyperbolic binding curve with a $K_d$ of 4 $\mu$M and a cross-linking efficiency ranging from 10% to 70% depending on conditions; second, ATP, dATP, and adenosine 5′-*O*-(3-thiotriphosphate) (ATP-$\gamma$-S) specifically inhibit the cross-linking of azido-ATP to recA protein; third, azido-ATP is a substrate for recA protein ATPase activity. Quantitative analysis of the cross-linking inhibition studies using a variety of nucleotide cofactors as competitors has shown that the binding affinity of adenine-containing nucleotides for recA protein decreases in the following order: ATP-$\gamma$-S > dATP > ATP > adenylyl $\beta,\gamma$-imidodiphosphate (AMP-PNP) $\gg$ adenylyl $\beta,\gamma$-methylenediphosphate (AMP-PCP) $\approx$ adenine. Similar competition studies also showed that nearly all of the other nucleotide triphosphates also bind to recA protein, with the affinity decreasing in the following order: UTP > GTP $\approx$ dCTP > dGTP > CTP. In addition, studies performed in the presence of single-stranded DNA demonstrated that the affinity of ATP, dATP, ATP-$\gamma$-S, and AMP-PNP for recA protein is significantly increased. These results are discussed in terms of the reciprocal effects that nucleotide cofactors have on the modulation of recA protein–single-stranded DNA binding affinity and vice versa. In addition, it is demonstrated that nucleotide and DNA binding are necessary though not sufficient conditions for ATPase activity. The significance of this result in terms of the possible requirement of critically sized clusters of 15 or more recA protein molecules contiguously bound to DNA for ATPase activity is discussed.

The recA protein of *Escherichia coli* is a DNA-dependent ATPase which has been shown to play an important role in the processes of genetic recombination and UV-inducible DNA repair [for reviews see McEntee and Winstock (1981), Radding (1982), Dressler and Potter (1982), and Little and Mount (1982)]. The enzymatic activities of this protein in vitro include the renaturation of complementary strands of single-stranded DNA (Winstock et al., 1979), the assimilation of complementary single-stranded DNA into duplex DNA molecules (Shibata et al., 1979; McEntee et al., 1979; West et al., 1981a,b), and the proteolytic cleavage of lexA and $\lambda$ repressor proteins (Craig & Roberts, 1980; Little et al., 1980). A requirement of each of these reactions is that recA protein must bind ATP (or a suitable ATP analogue), and in the case of the first two reactions, recA protein must hydrolyze ATP.

Although the exact mechanistic function of ATP binding and hydrolysis in these reactions is not yet understood, ATP and other nucleoside tri- and diphosphates have been shown to have a significant effect on the single-stranded DNA binding properties of recA protein (McEntee et al., 1981; Menetski

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& Kowalczykowski, 1985). Previous studies on the role of various nucleoside triphosphates in recA protein catalyzed reactions have focused primarily on the effects of these nucleotide cofactors on the enzymatic activities of recA protein, and due to the complexity of these reactions, molecular interpretations have been difficult [Weinstock et al., 1981a–d; see Weinstock (1982) for summary].

In order to develop a physical mechanistic understanding of the role of ATP binding and hydrolysis in these enzymatic reactions, it will be necessary to understand how recA protein interacts with each of its substrates and how these interactions are affected by the presence of other substrates. In this paper, the binding of nucleoside triphosphates and their analogues to recA protein is investigated in the absence and presence of DNA.

The approach employed was to use a photoaffinity analogue of ATP, azido-ATP[1] (Czarnecki et al., 1979, to study the binding of this analogue to recA protein through direct cross-linking. Numerous studies have previously demonstrated the usefulness of this ATP analogue in studies with various ATP binding and ATP hydrolytic proteins (King et al., 1982; Abraham et al., 1983; Hollemans et al., 1983; Woody et al., 1984; Abraham & Modak, 1984). Recently Knight and McEntee (1985a,b) demonstrated that azido-ATP cross-linking is specific for the ATP binding site of recA protein, and they have identified both the peptide fragment and amino acid residue that is cross-linked to the azido-ATP. The results presented in this paper are in agreement with the conclusion of Knight and McEntee (1985a,b) that azido-ATP cross-linking is specific for the ATP binding site of recA protein, and in addition, the results presented here both complement and extend the studies of Knight and McEntee (1985a,b) in the following quantitative and mechanistic ways. First, we hve quantified the binding of azido-ATP to recA protein and have used this knowledge, in turn, to determine the binding constants ($K_d$) of all of the nucleoside triphosphates to recA protein through competition studies; using estimates of the physiological concentrations of these nucleoside triphosphates in vivo, we have determined the likely relative proportions of the different nucleotide-bound forms of recA protein in vivo. Second, we have demonstrated that the affinity of recA protein for nucleoside triphosphates in the *presence* of DNA is increased; this property of recA protein is important to the understanding of the role of ATP binding in various DNA-dependent recA protein reactions. Third, we have utilized these binding parameters to demonstrate that ATP binding and ATP hydrolysis are not related in a one-to-one manner, but rather that a greater than 90% saturation of the recA protein–single-stranded DNA complex by ATP is required before ATP hydrolysis is observed; a possible molecular explanation providing insight into the mechanism of recA protein ATPase activity is discussed.

## Materials and Methods

*Chemicals and Buffers.* All chemicals used were reagent grade, and all solutions were made in glass-distilled water. The azido-ATP binding and cross-linking studies were carried out in a buffer consisting of 20 mM Tris-HCl, 4 mM $MgCl_2$, and 1 mM $\beta$-mercaptoethanol, at pH 7.5. RecA protein solutions used in the cross-linking experiments were dialyzed into the above buffer or into a buffer consisting of 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM $\beta$-mercaptoethanol, and 10% (v/v) glycerol, at pH 7.5, with no difference in the results obtained. All of the nucleotides except ATP-$\gamma$-S (Boehringer-Mannheim) were purchased from P-L Biochemicals, and all were dissolved in distilled water and adjusted to a final pH value of 7.

*Azido-ATP.* Initial samples of azido-ATP were obtained from Dr. Boyd Haley, University of Wyoming (currently at the University of Kentucky), to whom I am grateful for providing the azido-ATP as well as advice on its use. Subsequent batches of azido-ATP (both cold and $\gamma$-$^{32}$P-labeled) were purchased from New England Nuclear and showed no differences from the azido-ATP provided by Dr. Haley. However, attempts to use $\alpha$-$^{32}$P-labeled azido-ATP produced by Schwarz-Mann were unsuccessful for undetermined reasons related to the quality of the $\alpha$-$^{32}$P-labeled material. Stocks of the azido-ATP were stored either in methanol or were dried and stored in 20 mM Tris-HCl, pH 7.5. The integrity of the azido-ATP was checked by ultraviolet spectroscopy and by poly(ethylenimine)–cellulose thin-layer chromatography (see below). The azido-ATP concentration was determined by using a molar extinction coefficient of $1.33 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 281 nm.

*RecA Protein.* RecA protein was purified from strain KM1842 by using the procedure of Cox et al. (1981). The DNA–cellulose used in the procedure was prepared by the method of Litman (1968) using heat-denatured calf thymus DNA (Sigma). The protein concentration was determined by using a molar extinction coefficient of $2.7 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 280 nm.

*Ultraviolet Irradiation.* UV cross-linking of the azido-ATP to recA protein was achieved by using a "middle-wave" UV lamp (XX-15B) produced by Spectronics Corp. The lamp consists of two 15-W tubes that emit at wavelengths from ≈285 to 370 nm, with a maximum intensity at ≈300 nm. Irradiation of samples was generally from a distance of approximately 2–3 cm; the manufacturer's specifications indicate that the irradiance at 15 cm is 1800 $\mu W/cm^2$. The advantage that this middle-wave lamp has over a short-wave lamp ($\lambda_{max}$ = 254 nm) in these cross-linking experiments is that complications due to absorption of the incident light by added nucleotides during the competition experiments described in Results section D are eliminated. In addition, the absorption maximum of the azido-ATP molecule occurs at 281 nm (and trails to ≈320 nm) and, therefore, overlaps well with the lamp emission spectrum; the absorption spectrum of azido-ATP has a minimum near 254 nm.

Standard buffers for UV irradiation experiments were 20 mM Tris-HCl, 4 mM $MgCl_2$, and 1 mM $\beta$-mercaptoethanol, at pH 7.5; where indicated, either NaCl or DNA was added to this solution. Samples (typically 50 $\mu$L) were irradiated at room temperature (≈22 °C) in either microtiter plates or Eppendorf tubes. The irradiation time was typically 1 min for experiments performed in the absence of DNA and 15 s for experiments in the presence of DNA. In the absence of azido-ATP, no inactivation of protein enzymatic activity was observed under these conditions. Reactions were stopped by removing them from the light source and adding an equal volume of cold 20% trichloroacetic acid. Ninety microliters was applied to Whatmann GF/C or GF/A glass filter that was presoaked in 10% trichloroacetic acid and washed successively three times with 0.5 mL of 10% trichloroacetic acid and two times with 95% ethanol. Filters were dried under a heat lamp and then counted in 4a20 scintillation mixture

[1] Abbreviations: azido-ATP, 8-azidoadenosine 5′-triphosphate; AMP-PNP, adenylyl $\beta,\gamma$-imidodiphosphate; AMP-PCP, adenylyl $\beta,\gamma$-methylenediphosphate; ATP-$\gamma$-S, adenosine 5′-*O*-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

(Research Products International, Inc.) in a Beckmann LS-7800 scintillation counter. Nonlinear least-squares fitting of the binding data was performed on a Hewlett-Packar 86B computer using the software package "DAES80" (R & L Software Co.).

*ATPase Assay.* The hydrolysis of ATP or azido-ATP by recA protein at 37 °C was determined by separating the inorganic phosphate, ADP, and ATP on poly(ethyleneimine)–cellulose Thin-layer chromatography plates (Brinkmann) using 0.75 M sodium phosphate, pH 3.5, as the developing buffer. The single-stranded DNA used was heat-denatured calf thymus DNA (Sigma) at a concentration of 20 $\mu$M; the recA protein concentration was typically 1.0 $\mu$M. The [$^3$H]ATP used was purchased from ICN or from New England Nuclear. The extent of hydrolysis was determined by cutting out the appropriate spots and counting in 4a20 scintillation cocktail.

*Spin Columns.* When necessary, unreacted azido-ATP was separated from recA protein by using 1-mL spin columns (Neal & Florini, 1973) consisting of Bio-Gel P-6DG (Bio-Rad) equilibrated with 10 mM Tris-HCl and 1 mM EDTA, pH 7.5. The sample volumes applied were 0.1 mL, and >95% of the residual nucleotides were removed by using this procedure.

*Gel Electrophoresis and Autoradiography.* SDS–acrylamide gels containing 8 M urea were prepared according to the recipes in Maizels (1979). After electrophoresis, gels were Coomassie stained and photographed. They were then dried and autoradiographed at −20 °C with XAR-5 film (Kodak) and a Cronex intensifying screen (Du Pont).

To determine the specific activity of a labeled band on an autoradiograph, both the negative of the Coomassie stained gel and the autoradiograph were scanned by using a Zeneh laser scanning densitometer (Bio-Med Instruments) interfaced to a Hewlett-Packard 3390 recording integrator. Film exposures varying over a 5–10-fold range in exposure time were used to ensure that photographic response was linear. The specific activity of azido[$^{32}$P]ATP incorporation in a given peptide band was defined as the ratio (in arbitrary units) of the percent of total $^{32}$P incorporated into that band as determined from the autoradiograph to the percent of total protein in that band as determined from the Coomassie stained gel, and the ratio was normalized for molecular weight differences.

## RESULTS

*(A) Binding of Azido-ATP to RecA Protein.* The binding and photo-cross-linking of azido-ATP to recA protein is demonstrated in Figure 1. As the concentration of azido-ATP is increased, it is clear that the extent of photo-cross-linking to the protein approaches a plateau value indicative of saturation binding behavior. At higher concentrations of azido-ATP (i.e., >30–40 $\mu$M), a gradual linear increase in the amount of cross-linking is observed (not shown). This approximately linear increase in cross-linking labeling is characteristic of nonspecific photo-cross-linking and is typically observed at high concentrations (i.e., greater than ≈40 $\mu$M) of a photoreactive reagent [see Czarnecki et al. (1979) and Geahlen and Haley (1979) for further discussion of nonspecific binding]. At 20 $\mu$M azido-ATP, the amount of nonspecific binding is less than 10% of specific binding and consequently has little impact on the quantitative analysis of these data. Therefore, only the data in this range of azido-ATP concentration was used to determine the binding affinity of azido-ATP to recA protein.

The affinity of azido-ATP binding to recA protein can be determined by direct nonlinear least-squares fitting of the data in Figure 1 to a hyperbola. Fitting the raw data (not shown)

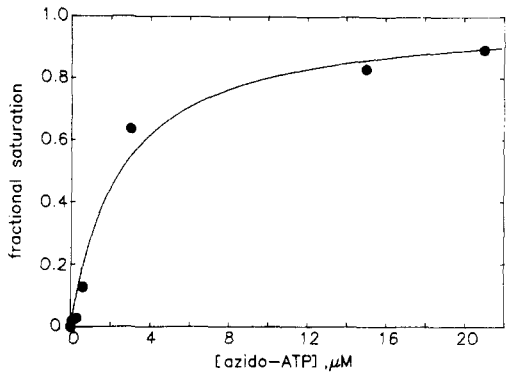


FIGURE 1: Concentration dependence of azido-ATP photo-cross-linking to recA protein. The concentration of recA protein is 1 $\mu$M, and cross-linking was performed by using standard conditions described under Materials and Methods. The solid line represents the best fit of the data to a hyperbola as given by the equation $y = 1.00x/(x + 2.49)$.

yields a value of 2710 (±219) for the cpm at saturation, and the data in Figure 1 have been normalized by dividing by 2710 cpm so that the $y$ axis is in terms of fractional saturation. In addition, the hyperbolic fit yields a value of 2.5 ± 0.8 $\mu$M for the total azido-ATP concentration required for one-half saturation of the protein. Since the dissociation constant, $K_d$, is defined as the free concentration of azido-ATP at one-half protein saturation, $K_d$ can be obtained by subtracting one-half of the total protein concentration (=0.5 $\mu$M), yielding a value of $K_d$ = 2.0 $\mu$M or a binding constant, $K_a$, of $5.0 \times 10^5$ M$^{-1}$.

The binding data in Figure 1 can also be recast into the form of a Scatchard plot (not shown) yielding a value of $K_a$ = (4.4 ± 0.7) × $10^5$ M$^{-1}$ which, as expected, is in agreement with the value determined from the midpoint of the binding curve. The average value of $K_a$ for azido-ATP binding, determined from four separate experiments at recA protein concentrations ranging from 1 to 3.7 $\mu$M, is (2.7 ± 2.0) × $10^5$ M$^{-1}$ or a value of $K_d$ = 3.7 $\mu$M.

The binding stoichiometry at saturation cannot be obtained from these data due to the fact that a quantity proportional to binding (i.e., cpm) is measured rather than the actual binding density. However, equilibrium dialysis data have shown that the stoichiometry of ATP binding under these conditions is one ATP molecule per recA protein monomer (Cotterill et al., 1982). In addition, Knight and McEntee (1985a,b) demonstrated that a unique site is cross-linked in the protein. Thus, it is assumed that the $K_a$ determined is for the binding of one azido-ATP molecule per recA protein molecule.

*(B) Time Course and Efficiency of Labeling.* In order to determine the optimal length of time for photoirradiation, a time course of recA protein labeling by azido-ATP was determined, and it was found that only 15 s of irradiation with the mid-wave light source was adequate for maximal photo-cross-linking (not shown). Photoirradiation times of as long as 10 min had no efect on the extent of labeling, indicating that photoactivated reversal of the chemical cross-linking was not occurring. Thus, in all the experiments that follow, an irradiation time of 1 min was normally used except in the experiments containing DNA, where an irradiation time of 15 s was employed.

The efficiency of azido-ATP cross-linking to recA protein was found to vary significantly depending on the concentration of $\beta$-mercaptoethanol present in the reaction buffer. On the basis of both calculations using the specific activity of the $^{32}$P-labeled azido-ATP employed and the amount of trichloroacetic acid precipitable radioactivity specifically

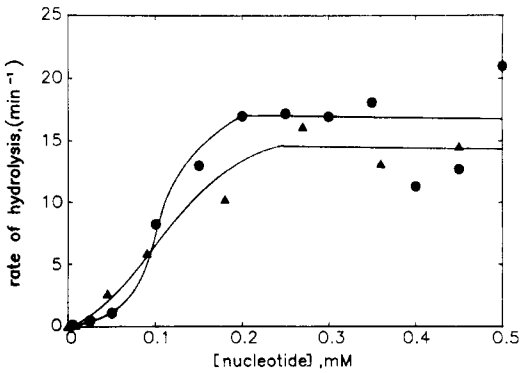


FIGURE 2: Dependence of recA protein nucleoside triphosphate hydrolytic activity on nucleoside triphosphate concentration. The rate of hydrolysis is given in terms of moles of ATP hydrolyzed per mole of recA protein per minute. The concentration of recA protein is 1 $\mu$M, and the concentration of heat-denatured calf thymus DNA is 5 $\mu$M. Hydrolysis was at 37 °C for 10 min: (●) azido-ATP; (△) ATP.

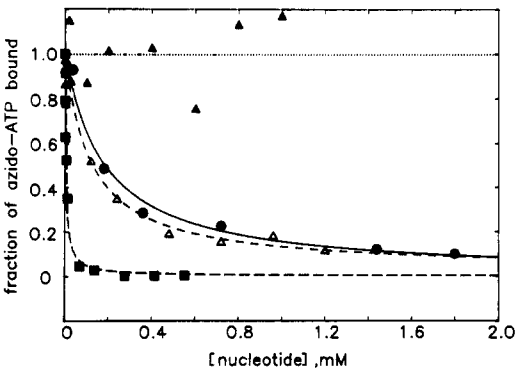


FIGURE 3: Effect of various adenine-containing nucleotides on the cross-linking of azido-ATP to recA protein. The concentration of recA protein is 1 $\mu$M, and the concentration of azido-ATP is 45 $\mu$M. The lines represent the best-fit hyperbola to each data set: (▲) AMP-PCP; (●) ATP; (△) dATP; (■) ATP-$\gamma$-S.

cross-linked to the protein, as well as on direct separation of the cross-linked protein from unreacted recA protein,[2] it was found that the efficiency of cross-linking varied from approximately 10–20% (i.e., moles of azido-ATP per mole of recA protein) at 1.0 mM $\beta$-mercaptoethanol to 60–80% at 0.1 mM $\beta$-mercaptoethanol.

In addition, inhibition of the single-stranded DNA-dependent ATPase activity of recA protein by azido-ATP cross-linking was also measured, and the inhibition was observed to generally parallel the extent of cross-linking; i.e., at 0.1 mM $\beta$-mercaptoethanol, the ATPase activity of recA protein was inhibited $\approx$70 $\pm$ 10% and at concentrations of $\beta$-mercaptoethanol ranging from 0.25 to 1.0 mM, the activity was inhibited $\approx$15 $\pm$ 5% relative to the activity of unirradiated protein. In control experiments, no inhibition of recA protein ATPase activity by UV irradiation was observed in either the presence or absence of ATP. Thus, the extent of cross-linking is approximately linearly related to the amount of ATPase activity inhibition. Consistent with this result is the observation that when purified cross-linked azido-ATP–recA protein is added to unmodified recA protein, no inhibition of the unmodified protein ATPase activity is seen.[2]

At all $\beta$-mercaptoethanol concentrations, the binding of azido-ATP to recA protein was approximately 90% specific for the ATP binding site, on the basis of competition experiments described below, suggesting that the bound azido-ATP is relatively accessible to chemical quenching by the $\beta$-mercaptoethanol. In all experiments to follow, 1.0 mM $\beta$-mercaptoethanol was employed to minimize the possibility that multiple binding and cross-linking events may be occurring at the lower $\beta$-mercaptoethanol concentrations.

(*C*) *Azido-ATP Is a Substrate for ATPase Activity.* The ability of azido-ATP to serve as a substrate for the recA protein DNA-dependent ATPase activity was determined to serve as an additional control of the properties of this ATP analogue with regard to recA protein enzymatic activity and to determine the $K_m$ value of the azido-ATP for comparison with published values of the $K_m$ for ATP (Weinstock et al., 1981b). As was shown by Knight and McEntee (1985a), azido-ATP is indeed a substrate for recA protein ATPase activity, and here we determine the $K_m$ for azido-ATP. The apparent $K_m$ for azido-ATP and ATP binding to recA protein can be obtained from a plot of the moles of ATP (or azido-ATP) hydrolyzed per minute per mole of recA protein vs. the nucleotide concentration, which is shown in Figure 2. As noted by Weinstock et al. (1981b), the binding of ATP to recA protein is apparently cooperative in ATP concentration, which is also the case for azido-ATP binding. The apparent $K_m$ values as determined from the midpoint to these sets of data is approximately 90 and 130 $\mu$M for azido-ATP and ATP, respectively. Thus, the binding of azido-ATP to recA protein as measured by the value of the $K_m$ for ATPase activity is approximately 50% greater than that of ATP. The value of $V_{max}$/recA protein concentration in the presence of azido-ATP is similar, though slightly greater than the value obtained in the presence of ATP. These values are in good agreement with those obtained by Weinstock et al. (1981b) and further confirm the fact that azido-ATP is good ATP analogue with regard to recA protein ATPase activity.

(*D*) *Competition of Azido-ATP Cross-Linking by ATP.* The final criteria for suitability of a substrate analogue for use in binding site studies in whether the natural substrate is an effective competitor of cross-linking by the analogue. Figure 3 (filled circles) shows the effect of increasing concentrations of normal ATP on the cross-linking of azido-ATP to recA protein. Clearly, ATP inhibits the binding and cross-linking of azido-ATP to recA protein, and as expected, the inhibition curve is hyperbolic. Absorption of the incident irradiating light with increasing ATP concentration is not a problem due to the use of the mid-wave light source.

The binding constant of ATP to recA protein can be obtained from the competition data shown in Figure 3 if two assumptions are made: first, the lifetime of the photoactivated azido-ATP intermediate is shorter than the dissociation time of the azido-ATP; second, molecules of activated azido-ATP not bound to recA protein are effectively quenched by reaction with the buffer components and subsequently do not react with the proteins. Given that nitrene intermediates generally (though not always) have a very short lifetime, on the order of milliseconds (Staros, 1980), and are also highly reactive, these are not unreasonable assumptions. It is not essential that the efficiency of cross-linking is 100%, only that the amount of azido-ATP cross-linked to the protein is proportional to the amount of azido-ATP bound to the protein at the time of UV irradiation. Thus photoirradiation of a solution of azido-ATP, recA protein, and competitor should result in an instantaneous representation of the proportion of azido-ATP molecules bound to recA protein. Consistent with these assumptions, Abraham and Modak (1984), Geahlen and Haley (1979), and Woody et al. (1984) have shown that using azido-ATP (or azido-GTP) cross-linking experiments to determine affinity constants for their respective systems yields results which are in agreement

[2] Kowalczykowski, unpublished observations.

Table I: Binding Parameters of Nucleotides to RecA Protein[a]

| nucleotide | $K_d$ ($\mu$M) | nucleotide | $K_d$ ($\mu$M) |
|---|---|---|---|
| azido-ATP | 3.7 ± 2.5 | adenine | $\gg 100$[b] |
| ATP | 15 ± 3 | GTP | 33 ± 4 |
| dATP | 10 ± 5 | dGTP | 50 ± 11 |
| ATP-$\gamma$-S | <0.3 ± 0.1 | CTP | $\gg 100$[b] |
| AMP-PNP | 25 ± 5 | dCTP | 34 ± 10 |
| AMP-PCP | $\gg 100$[b] | UTP | 13 ± 5 |

[a] The value of $K_d$ for azido-ATP was determined by direct titration experiments. Values of $K_d$ for the other nucleotides were obtained from competition experiments as described in the text; a value of $K_{d,azido\text{-}ATP}$ of 3.7 $\mu$M was used. The experimental error of $K_d$ reflects the larger value of either the standard deviation from fit or the variation between multiple experiments. [b] No inhibition was detectable at nucleotide concentrations up to 1 mM when azido-ATP was present at 45 $\mu$M.

with those obtained from equilibrium experiments. Since the concentration of azido-ATP used in Figure 3 is 45 $\mu$M, which is more than 10-fold above the average measured $K_d$ of 3.7 $\mu$M, the dissociation constant for ATP can be determined with good approximation from the concentration of ATP required to compete one-half of the azido-ATP binding by using the relationship

$$[\text{azido-ATP}]/K_{d,\text{azido-ATP}} = [\text{ATP}]/K_{d,\text{ATP}}$$

A nonlinear least-squares fit of these data yields an azido-ATP binding inhibition midpoint of 184 ± 41 $\mu$M, which yields a $K_d$ value for ATP binding of 15 ± 3 from the relationship above. Cotterill et al. (1982) have measured the binding of ATP to recA protein using a nonequilibrium dialysis method and have obtained values of $K_d$ ranging from 23 to 27 $\mu$M. Given the differences between this method and the azido-ATP competition method, the agreement is very good and serves to confirm that the assumptions made above are reasonable. Thus nucleotide dissociation constants can be obtained by using this azido-ATP competition procedure.

(*E*) *Effect of Other Nucleotide Cofactors.* Since nucleotide cofactors are known to have dramatic effects on the DNA binding properties and enzymatic activities of recA protein (McEntee et al., 1981; Phizicky & Roberts, 1981; Menetski & Kowalczykowski, 1985), the binding of a variety of adenine-containing nucleotide cofactors and cofactor analogues was determined by using the azido-ATP cross-linking competition method described above. The results are also shown in Figure 3, and it is clear that at one extreme ATP-$\gamma$-S is a highly effective inhibitor of azido-ATP binding, while at the other extreme AMP-PCP and adenine (not shown) show no inhibition of binding. These results, particularly the latter ones, confirm that inhibition of azido-ATP binding is a competitive effect specific for each nucleotide rather than the result of some nonspecific effect such as absorption of UV light by the added competitor. Values of $K_d$ obtained as described in the previous section are presented in Table I. Previous studies have demonstrated that ATP-$\gamma$-S binds very tightly to recA protein (Weinstock et al., 1981c), and these competition studies are in agreement, showing that $K_d$ for ATP-$\gamma$-S binding is less than 0.3 $\mu$M. The other ATP analogues, AMP-PNP and AMP-PCP, bind much more weakly than either ATP-$\gamma$-S or the natural substrates. These analogues do not support any ATP-requiring enzymatic activity of recA protein, but recent DNA binding studies have shown that high concentrations (>300 $\mu$M) of AMP-PNP (but not AMP-PCP) will induce a high DNA affinity form or recA protein that is much like the form induced by ATP or ATP-$\gamma$-S binding (Menetski & Kowalczykowski, 1985). Thus, these azido-ATP competition studies are in agreement with the DNA binding studies, and they suggest that the effectiveness of the various ATP analogues to induce a high DNA affinity state of recA protein is related to their intrinsic binding affinity to recA protein. As shown in Table I, there is a clear hierarchy of adenine nucleotide binding affinities: ATP-$\gamma$-S > azido-ATP > dATP > ATP > AMP-PNP $\gg$ AMP-PCP $\approx$ adenine.

Table II: Salt Dependence of Azido-ATP Binding Constants

| without single-strand DNA | | with single-strand DNA | |
|---|---|---|---|
| [NaCl] (mM) | $K_d$ ($\mu$M) | [NaCl] (mM) | $K_d$ ($\mu$M) |
| 0 | 3.7 ± 2 | 0 | 1.5 ± 0.6 |
| 100 | 35 ± 8 | 100 | 8 ± 2 |
| 200 | 60 ± 10 | 200 | 35 ± 7 |
| | | 500 | 100 ± 10 |

Similar competition data (not shown) can be obtained for the remaining nucleoside triphosphate, and the results are also presented in Table I. As can be seen from the values of $K_d$, each of these triphosphates (except CTP) binds to recA protein with an appreciable affinity. Since these nucleoside triphosphates are also present under physiological conditions, their effect on recA protein affinity should not be overlooked.

(*F*) *Effect of DNA and Salt Concentration on Azido-ATP Binding.* In addition to nucleotide cofactors, salt concentration also has a significant effect on recA protein–DNA binding affinity and recA protein enzymatic function (Weinstock et al., 1981a: Menetski & Kowalczykowski, 1985). Thus, the effect of increasing NaCl concentration on the binding of azido-ATP to recA protein was examined. When a competition experiment similar to those shown in Figure 3 was performed with NaCl as a competitor, it was found that increasing NaCl concentrations significantly inhibit the binding of azido-ATP to recA protein (not shown). Binding is half-inhibited at $\approx$100 mM NaCl, and complete inhibition occurs at concentrations of NaCl greater than $\approx$350 mM NaCl, indicating that under these experimental conditions the $K_d$ for azido-ATP binding must have increased to greater than $\approx$400 $\mu$M.

To ensure that this inhibition by NaCl was due to an actual inhibition of binding binding rather than to an effect on the efficiency of the photochemistry involved, the concentration dependence of azido-ATP binding at various salt concentrations was also determined (not shown). If the effect of NaCl were on the efficiency of cross-linking rather than on the actual binding affinity, then the data would have the identical shape as that in Figure 1, but the plateau values would decrease. Instead, the effect of increasing NaCl concentration is to shift the binding curve to the right, which is characteristic of weaker binding. The values of $K_d$ obtained at each of these different NaCl concentrations is summarized in Table II.

To determine whether the presence of single- or double-stranded DNA had any effect on the binding and cross-linking of azido-ATP to recA protein, a competition experiment similar to those described above was carried out by using DNA as the competitor. The results demonstrated that there is a slight ($\approx$15%) but detectable competitive effect due to addition of either type of DNA. This slight inhibitory effect may be due to hydrolysis of the $\gamma$-$^{32}$P label of the azido-ATP molecule rather than a direct competitive effect, but as will be shown below, it is more likely to be due to inhibition of azido-ATP cross-linking at a DNA binding site. One surprising result of these experiments is that despite activation of the recA protein ATPase activity by the presence of single-stranded DNA, most of the $\gamma$-$^{32}$P label remains on the cross-linked recA protein–Azido-ATP complex. Perhaps the cross-linking event inactivates the ATPase activity of the protein, and preliminary

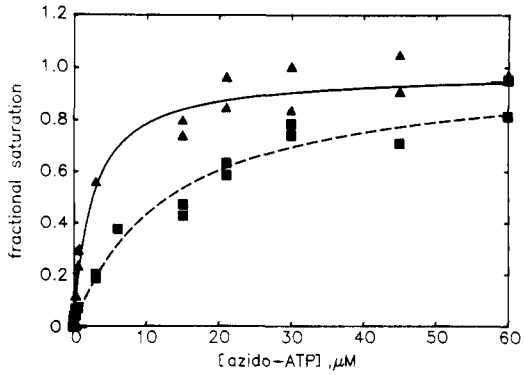


FIGURE 4: Concentration dependence of azido-ATP photo-cross-linking to recA protein in the presence of DNA. The concentration of recA protein is 1 μM, and that of DNA is 5 μM; photoirradiation was for 15 s: (▲) single-stranded DNA; (■) double-stranded DNA. The curves represent the best-fit hyperbola to each data set.

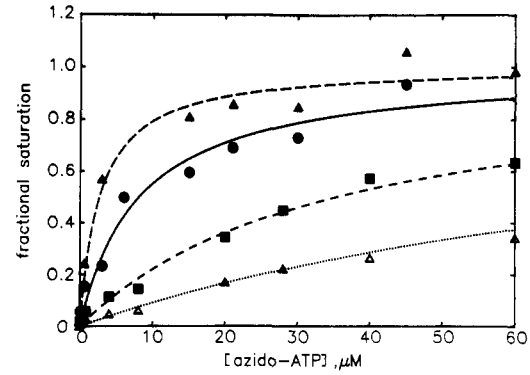


FIGURE 5: Effect of NaCl on azido-ATP binding to recA protein in the presence of single-stranded DNA. The concentration of recA protein is 1 μM, and that of the DNA (heat-denatured calf thymus DNA) is 5 μM: (▲) 0 mM NaCl; (●) 100 mM NaCl; (■) 200 mM NaCl; (△) 500 mM NaCl. The curves represent the best-fit hyperbola to each data set.

experiments are consistent with this interpretation;[2] this result permits the direct determination of nucleotide binding constants in the presence of DNA, and the results are described below.

(*G*) *Azido-ATP Binding in the Presence of DNA.* The binding of azido-ATP to recA protein in the presence of single- or double-stranded DNA is shown in Figure 4. It is clear in both cases that the binding and cross-linking of the azido-ATP to recA protein follows the expected hyperbolic curve. The data obtained in the presence of DNA (either single or double stranded) does not show the nonspecific binding at higher azido-ATP concentrations that is present in the DNA-free experiment. This result implies that either there is some very weak binding of the azido-ATP to the DNA binding site of recA protein or the binding of recA protein to DNA results in the elimination of a weak (perhaps fortuitous) ATP binding site. The significance of the site, if any, is not clear, but the elimination of this "nonspecific" binding allows for an accurate determination of the azido-ATP binding affinity in the presence of DNA. The values of $K_d$ obtained from the data in Figure 4 are 1.5 ± 0.6 and 11.5 ± 3 μM in the presence of single-stranded DNA and double-stranded DNA, respectively. Note that the affinity of azido-ATP binding to recA protein is 2.5-fold greater in the presence of single-stranded DNA than in its absence. This is expected on thermodynamic grounds since the binding of single-stranded DNA to recA protein is increased by the binding of ATP (Menetski & Kowalczykowski, 1985), and therefore the reciprocal effect should be observed.

To determine whether NaCl had the same effect on azido-ATP binding in the presence of single-stranded DNA that it has in its absence (see section F), azido-ATP binding experiments were carried out in 100, 200, and 500 mM NaCl, and the results are shown in Figure 5. These results are similar to those obtained in the absence of DNA, except that the binding of azido-ATP at a given NaCl concentration is always greater when single-stranded DNA is present (Table II), again confirming the fact that the binding of azido-ATP is positively linked to the binding of single-stranded DNA.

(*H*) *Competition Experiments in the Presence of Single-Stranded DNA.* Competition experiments similar to those described in section D were employed to determine the affinity of the natural nucleotides ATP and dATP and of the triphosphate analogues ATP-γ-S and AMP-PNP in the presence of single-stranded DNA. The competition curves observed (not shown) were similar to those shown in Figure 3, and the data were analyzed as described in section D. The results obtained are summarized in Table III. A comparison with the results

Table III: Binding Parameters of Nucleotides to RecA Protein in the Presence of Single-Stranded DNA[a]

| nucleotide | $K_d$ (μM) |
|---|---|
| azido-ATP | 1.5 ± 0.6 |
| ATP | 2.5 ± 1 |
| dATP | 1.1 ± 0.3 |
| ATP-γ-S | <0.4 ± 0.2 |
| AMP-PNP | 15 ± 4 |

[a] The footnote to Table I also applies to this table, except that a value of $K_{d,azido\text{-}ATP}$ of 1.5 μM was used. The concentration of heat-denatured calf thymus DNA was 5 μM, and the concentration of recA protein was 1 μM.

obtained in the absence of DNA (Table I) shows that in each case the nucleoside triphosphate binding affinity is greater in the presence of single-stranded DNA than in its absence. Thus, as mentioned previously with regard to azido-ATP binding, the binding of recA protein to single-stranded DNA induces a tight nucleotide triphosphate binding form of the protein.

(*I*) *Identification of Partial Proteolytic Peptides of RecA Protein That Bind Azido-ATP.* Two additional applications in which these azido-ATP binding and cross-linking studies are useful are the identification of the ATP binding site of recA protein [as was demonstrated by Knight and McEntee (1985a,b)] and the identification of partial proteolysis products of recA protein that retain the ability to bind ATP (as demonstrated below). The experiments designed to demonstrate these uses were carried out as follows. To identify which peptide fragments were involved in the ATP binding site of recA protein, the protein was cross-linked to azido-ATP, subject to partial digestion by trypsin (parts A and B of Figure 6, lanes 2–5 in each), and then run on an SDS–polyacrylamide gel and autoradiographed. To determine whether any of these partial proteolytic digestion peptide fragments retain the ability to bind azido-ATP, a similar experiment was carried out, except that the order was reversed; i.e., recA protein was first partially digested by trypsin (parts A and B of Figures 6, lanes 6–9 in each), cross-linked to azido-ATP, and then run on an SDS–polyacrylamide gel and autoradiographed.

The results of the tryptic digests in Figure 6 (lanes 2–5) show that when azido-ATP is added prior to proteolysis there are four predominant peptides (molecular masses of 40, 28, 17, and 9 kDa) as well as many minor bands that are labeled by azido-ATP; however, the relationship of each peptide to the others cannot be determined from these data. A more significant result is obtained when azido-ATP is added *after* proteolysis (lanes 6–9); only one of these peptides (28 kDa)

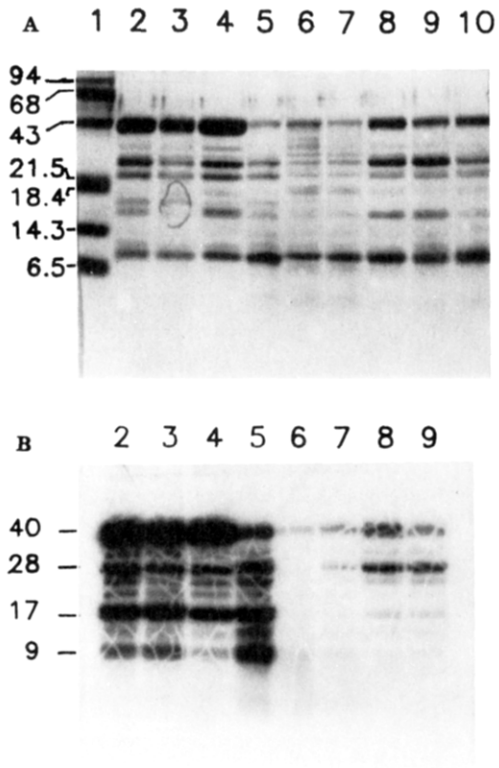


FIGURE 6: Tryptic digests of azido-ATP–recA protein complexes: (A) Coomassie stained gel; (B) autoradiographed of dried gel. Digests were carried out in standard reaction buffer at 37 °C at the following concentrations: [recA protein] = 10 $\mu$M; [azido-ATP] = 40 $\mu$M; [DNA] = 50 $\mu$M; [trypsin] = 10 $\mu$g/mL. Digestion was stopped with PMSF, and 25 $\mu$L was loaded on a 15% SDS–urea acrylamide gel. In lanes 2–5, recA protein was cross-linked to azido-ATP prior to tryptic digestion; in lanes 6–9 recA protein was first digested in either the presence or absence of DNA and was then cross-linked to azido-ATP. Lane 1 molecular weight standards: aprotinin (6.5 kDa); lysozyme (14.3 kDa); $\beta$-lactoglobulin (18.4 kDa); soybean trypsin inhibitor (21.5 kDa); ovalbumin (43 kDa); bovine serum albumin (68 kDa); phosphorylase *b* (94 kDa). Lanes 2 and 6, 3-min digest in the absence of DNA. Lanes 3 and 7, 6-min digest in the absence of DNA. Lanes 4 and 8, 3-min digest in the presence of heat-denatured calf thymus DNA. Lanes 5 and 9, 6-min digest in the presence of heat-denatured calf thymus DNA. Lane 10, control digest in the absence of DNA and azido-ATP.

is prominently labeled (in addition to the 40-kDa peptide that is intact recA protein), indicating that only this partial proteolytic product retains the ability to bind azido-ATP and, by inference, ATP. When the specific activity of labeling (i.e., the intensity from the autoradiograph/intensity from Coomassie stained gel) of this fragment is compared to that of the intact recA protein, a similar value is obtained, suggesting that azido-ATP binding to this fragment of recA protein is not significantly attenuated. This fragment has the prospects, therefore, of retaining some partial activity of the intact recA protein and might be useful in studies aimed at dissecting the enzymatic mechanism of recA protein function; efforts are currently under way to isolate it in native form. The 17-kDa fragment is able to bind azido-ATP also, although its yield is too low to determine the specific activity of labeling with any accuracy.

## Discussion

In this paper it has been demonstrated that azido-ATP is a good analogue of ATP for use in recA protein–nucleotide binding studies. The suitability of this ATP analog to effectively substitute for normal ATP, with regard to recA protein binding and enzymatic function, has been demonstrated in three ways. First, direct binding and photo-cross-linking studies of azido-ATP to recA protein have shown that this analogue binds to recA protein with a dissociation constant, $K_d$, of $\approx$4 $\mu$M in the absence of DNA. This cross-linking follows a simple hyperbolic binding curve, and the extent of binding shows saturation behavior particularly in the presence of single-stranded DNA. These results are indicative of specific binding and cross-linking of the azido-ATP to recA protein. This conclusion is in agreement with the work of Knight and McEntee (1985a,b), who demonstrated that a specific amino acid residue (tyrosine 264) within a unique tryptic peptide of recA protein is the covalent attachment site for the azido-ATP molecule.

Second, the specific binding of azido-ATP to recA protein can be completely inhibited by nucleotides that are known to bind to recA protein, i.e., ATP, dATP, and ATP-$\gamma$-S. Analysis of the results obtained from azido-ATP cross-linking inhibition by competition with ATP yields a value for $K_d$ of 15 $\mu$M for ATP binding to recA protein. This value is in excellent agreement with the value of $K_d$ ($\approx$24 $\mu$M) obtained by Cotterill et al. (1982) using a nonequilibrium dialysis technique. This agreement further confirms the assumption that these competition studies can be used to quantitatively investigate the binding of other nucleotides to recA protein.

Third, azido-ATP is utilized effectively by recA protein as a substrate for ATPase activity. Both the time course and the nucleotide concentration dependence of azido-ATP hydrolysis are similar to that of ATP hydrolysis. The ATP hydrolytic activity for both nucleotides shows the sigmoidal dependence on nucleotide concentration that has been characterized by Weinstock et al. (1981b), and the apparent $K_m$ values obtained from Figure 3 are $\approx$90 and $\approx$130 $\mu$M for azido-ATP and ATP at pH 7.5, respectively. Weinstock et al. (1981b) reported that the $K_m$ values for recA protein ATPase activity stimulated by heat-denatured calf thymus DNA ranged from 67 $\mu$M at pH 8.1 to 103 $\mu$M at pH 6.2 (though values as low as 20 $\mu$M were obtained with $\phi$X174 single-stranded DNA); the $K_m$ values reported here with calf thymus DNA are in general agreement with the previously reported values. In addition, the $K_m$ values determined here for azido-ATP vs. ATP demonstrate that azido-ATP has a higher apparent affinity (i.e., lower $K_m$) for recA protein than does ATP, which is in qualitative agreement with the direct binding results mentioned above. Note, however, that the numerical values of $K_d$ and $K_m$ are not identical either for azido-ATP or ATP. These numbers would only be identical if the kinetic mechanism of ATP hydrolysis were a simple process. From the studies reported by Weinstock et al. (1981a,b), the enzymatic properties of the ATP hydrolysis reaction are quite complex, and therefore numerical agreement of the $K_m$ and $K_d$ values would not be expected.

Although the specific mechanism and function of ATP hydrolysis is not known, a comparison of the data in Figures 2 and 4 provides interesting insight into the nature of the cooperative behavior of recA protein ATPase activity. For clarity, these data have been replotted in Figure 7, where their *y* axes have been normalized to represent the fraction of maximal binding (data from Figure 4) or fraction of maximal ATPase activity (data from Figure 2). When presented in this manner, it is readily evident that a significant amount of ATP binding occurs at nucleotide concentrations that are well below those required for stimulation of ATPase activity. Less than 10–20% of the maximum ATPase activity is observed at a nucleotide concentration (50 $\mu$M) where greater than 90% of the azido-ATP or ATP is bound, and maximum ATPase activity does not occur until $\approx$99% of the recA protein is com-

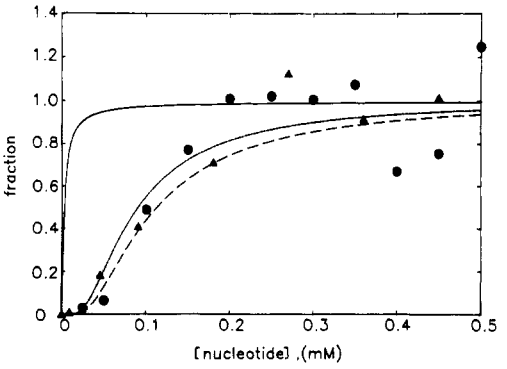


FIGURE 7: Fraction of maximum ATPase activity and nucleotide binding as a function of nucleotide concentration. The data from Figure 2 were normalized to represent the fraction of maximum ATPase activity by dividing by 16.8 $min^{-1}$ and 14.3 $min^{-1}$ for the azido-ATP (●) and ATP (▲) data, respectively. The solid line represents the fraction of recA protein that is bound to ATP and was determined by using a value of $K_{d,ATP}$ = 2.5 $\mu$M (Table III); the fraction of azido-ATP-bound recA protein was determined by using a value of $K_{d,azido\text{-}ATP}$ = 1.5 $\mu$M and is not plotted. The dashed curve (for the ATP data) and the dash-dot curve (for the azido-ATP data) represent the calculated fractional saturation of the single-stranded DNA by clusters of nucleotide-bound recA protein that are greater than $C$. The parameters used for the theoretical curves are $n$ = 5, $\omega$ = 1, and $C$ = 15 (dashed) and $n$ = 5, $\omega$ = 1, and $C$ = 20 (dash-dot).

plexed with nucleotide. This comparison clearly demonstrates that nucleotide binding to a complex of recA protein and DNA is a necessary though not sufficient requirement for ATPase activity, and, therefore, the cooperative dependence of ATPase activity on nucleotide concentration is not the result of cooperative ATP binding. Although a variety of potential mechanisms exists to explain the cooperative nature of the ATPase activity, one possible explanation that will be discussed here is that recA protein ATPase activity requires the formation of *clusters* of ATP–recA protein molecules that are bound contiguously along the single-stranded DNA and that an individual recA protein molecule becomes active in ATPase activity only when it is part of a cluster that has exceeded a certain size. The cluster size distribution of protein molecules bound to DNA can be determined by using the formalism of McGhee and von Hippel (1974), and the pertinent equations are described elsewhere (Kowalczykowski et al., 1986). From these equations, the critical cluster size ($C$) required for ATP hydrolysis can be determined if it is assumed that the fraction of maximal ATPase activity at any given nucleotide concentration is proportional to the fraction ($F$) of the DNA-bound recA protein molecules that are contained in clusters ($C$) of contiguously bound ATP–recA protein–DNA complexes that are greater than some critically sized cluster essential for ATPase activity and that clusters smaller than $C$ have no ATPase activity. (Details of these calculations and further discussion of the assumptions and limitations will appear elsewhere.[3] )

In Figure 7, this fraction, $F$, is plotted by using values for the parameters (i.e., site size, $n$, and the cooperativity, $\omega$) that yielded the *minimum* value for $C$ that fit the data; higher values of $\omega$ would have required greater values of $C$ to fit the data (i.e., using $\omega$ = 50 would require that $C \approx 50$).[3] Although higher values of $\omega$ might be more appropriate for use in this analysis, the intent here is to demonstrate that a minimum critical cluster size for ATPase activity may exist and to define the *minimum* value for that cluster. The calculated curves show a sigmoidal dependence on nucleotide concentration that is similar in appearance to the ATPase activity curve, and there is reasonably good agreement with the ATPase activity data for the calculated curves when $\omega$ = 1 and $C$ = 15–20 (or $\omega$ = 50, $C \approx 50$). Thus, if one assumes that this type of analysis of the ATPase activity data is valid, these results would indicate that in addition to ATP and DNA binding a critical cluster of at least 15 or more contiguously DNA–bound ATP–recA protein molecules is necessary for ATPase activity. Clearly, further experimental evidence will be required to determine whether the assumptions made in this analysis are correct, but until that time, this analysis provides for an interesting molecular interpretation of the sigmoid ATPase activity and may further suggest that such a critical cluster is important in more complex reactions such as strand assimilation. Consistent with these ideas are the observations that oligo $(dT)_{12}$ does not support recA protein ATPase activity, whereas a 74-nucleotide single-stranded DNA fragment does (Weinstock et al., 1981a). Also interesting is the observation that recA protein will catalyze homologous pairing between duplex DNA and single-stranded DNA molecules that share 151 nucleotides of homology but not between molecules that share only 30 nucleotides of homology (Gonda & Radding, 1983), though the exact relationship between this activity of recA protein and its ATPase activity is unclear.

The nucleotide competition studies performed in the absence of single-stranded DNA demonstrate that almost all of the nucleoside triphosphates show some affinity for recA protein (see Table I), despite the fact that may of them are not hydrolyzed by recA protein (Weinstock et al., 1981a). Among the adenine-containing nucleotides, ATP-$\gamma$-S has the greatest affinity for recA protein with an affinity that is too high to measure accurately. Thus, an upper limit of $K_d$ for ATP-$\gamma$-S binding is estimated to be less than 0.3 $\mu$M. Previous studies have demonstrated that ATP-$\gamma$-S forms a very stable complex with recA protein in the presence of single-stranded DNA (Craig & Roberts, 1981; Weinstock et al., 1981c) and that it inhibits the ATPase activity of recA protein with a $K_i$ of approximately 0.6 $\mu$M (Weinstock et al., 1981c). In this paper, the values obtained for $K_d$ in the presence and absence of single-stranded DNA are both <0.4 $\mu$M, which agree well with the reported value of $K_i$ for ATP-$\gamma$-S. For the other adenine nucleotides, the order of increasing binding affinity is dATP > ATP > AMP–PNP ≫ AMP–PCP ≈ adenine. The two latter nucleotides show no competitive effect on azido-ATP cross-linking and thus serve as controls demonstrating that absorption of the incident irradiating light is not a problem in these competition experiments. The higher affinity of dATP for recA protein over ATP is more apparent in the experiments performed in the presence of single-stranded DNA. These results are consistent with published measurements of the $K_m$ values for dATP and ATP obtained from ATPase activity studies that show a 2.5-fold higher $K_m$ value for ATP compared to dATP (Phizicky & Roberts, 1981).

The values of $K_d$ in Table I also reveal that all of the naturally occurring nucleoside triphosphates (except CTP) binding to recA protein with an appreciable affinity. This result suggests that, in vivo, a certain percentage of recA protein will be bound to these other nucleoside triphosphates. The actual amount of each nucleoside triphosphate–recA protein complex will be proportional to the product of $K_{a,NTP}$ and $L_{NTP}$, the free nucleoside triphosphate concentration. If it is assumed that the concentrations of the nucleotide triphosphates within *E. coli* are similar to those reported for *Salmonella typhimurium* (Bochner & Ames, 1982), then the values in Table I can be used to calculate the relative amounts

[3] Kowalczykowski, unpublished results.

Table IV: Relative Amounts of RecA Protein–Nucleoside Triphosphate Complexes

| nucleotide | % of recA protein–nucleotide complex[a] |
|---|---|
| ATP | 63 |
| dATP | 5 |
| GTP | 9 |
| dGTP | 1 |
| CTP | 0 |
| dCTP | 1 |
| UTP | 22 |

[a] Determined by using the values of $K_d$ in Table I and the nucleoside triphosphate concentrations determined by Bochner and Ames (1982).

of each nucleoside triphosphate–recA protein complex present in vivo, and the results are shown in Table IV. As might be expected, the ATP–recA protein complex is the predominant form of the protein in vivo; however, it represents only 63% of the total nucleotide-complexed protein present. Interestingly, 22% of the recA protein is expected to be complexed with UTP, a nucleotide that is readily hydrolyzed by recA protein but does not support the strand assimilation reaction (Weinstock et al., 1981d); however, UTP does partially support the proteolysis of λ repressor protein by recA protein (Weinstock & McEntee, 1981), and therefore this result may be relevant to recA protein's role in SOS induction. The fact that the non-ATP and non-dATP recA protein complexes represent ≈32% of the recA–nucleotide complexes in vivo may be significant, since many of these nucleoside triphosphates are not hydrolyzed by recA protein (Weinstock et al., 1981a) and none of them, other than ATP or dATP, support its strand assimilation activity. Also significant is the fact that some of the nucleoside triphosphates (except ATP and dATP) lower the affinity of recA protein for single-stranded DNA,[4] and this latter result may be important from the standpoint of the processivity of the recA protein catalyzed strand assimilation reaction. If the premature dissociation of one or more recA protein molecules from single-stranded DNA by non-adenine-containing nucleotide triphosphates has an effect on the extent of strand assimilation, then the reaction in vivo may not be as efficient as that observed in vitro. Experiments are in progress to determine whether such an effect is observed in vitro.

The data in Table II demonstrate that the binding of azido-ATP to recA protein is salt-dependent, decreasing to a value of $K_d \approx 60$ μM at 200 mM NaCl. This concentration of NaCl approximately represents the estimated effective in vivo salt concentration within *E. coli* (Kao-Huang et al., 1977). Since the ATP concentration in vivo is estimated to be ≈3 mM, recA protein will always be found in vivo complexed with ATP (or some other nucleoside triphosphate, as discussed in the previous paragraph). This result lends support to the inference that the first step in the recA protein–single-stranded DNA binding cycle proposed by Menetski and Kowalczykowski (1985) involves the binding of ATP to recA protein, which is then followed by binding of the ATP–recA protein complex to single-stranded DNA. Direct kinetic studies are in progress to test this hypothesis in vitro.

A comparison of the data in Tables I and III shows that the affinity of recA protein for all of the nucleoside triphosphates and triphosphate analogues is greater by as much as 10-fold in the presence of single-stranded DNA than in its absence. This fact is also confirmed by the salt-dependence data in Table II which show that the affinity of azido-ATP for recA protein is 2–4-fold greater in the presence of single-stranded DNA compared to in the absence of DNA at salt concentrations ranging from 0 to 200 mM NaCl. It has been demonstrated that ATP, ATP-γ-S, and AMP-PNP increase the affinity of recA protein for single-stranded DNA (Menetski & Kowalczykowski, 1985). Therefore, elementary thermodynamic considerations would predict that single-stranded DNA binding to recA protein would, in turn, increase the affinity of recA protein for ATP, ATP-γ-S, and AMP-PNP, a prediction which is borne out by the data presented in this paper. The data in Tables I and III would require that ATP binding to recA protein increase the protein's affinity for single-stranded DNA by 6-fold at these buffer conditions and that dATP or AMP-PNP binding result in a 9-fold and 2-fold increased single-stranded DNA affinity, respectively. Unfortunately, quantitative DNA binding data at this low salt concentration is not available to confirm this thermodynamic prediction. Menetski and Kowalczykowski (1985) also observed that ATP-γ-S was the most effective in increasing the affinity of recA protein for single-stranded DNA, followed by ATP and AMP-PNP, and the data in Table III demonstrate that this is the same order of nucleotide binding affinity to recA protein. Thus these azido-ATP binding competition studies confirm the proposal made by Menetski and Kowalczykowski (1985) that ATP binding increases the affinity of recA protein for single-stranded DNA and that a coupled equilibrium exists between ATP binding and single-stranded DNA binding.

An additional interesting observation based on the data in Figure 4 is that, in contrast to the effect of single-stranded DNA, the binding affinity of azido-ATP to recA protein does not increase in the presence of double-stranded DNA. This result implies that the reciprocal effect described above may not occur with duplex DNA, namely, the azido-ATP (or ATP) does not increase the affinity of recA protein to double-stranded DNA or that it does to a lesser (nondetectable) degree. However, DNA binding studies of recA protein carried out by McEntee et al. (1981) and in this laboratory[5] have shown that the kinetics of binding to duplex DNA are much slower. In addition, recent studies of the ATPase activity of recA protein stimulated by duplex DNA have demonstrated that the kinetic properties of this reaction are unusual, showing lag times up to several minutes long (Kowalczykowski, 1985).[6] In the studies presented here, photoirradiation of the azido-ATP–recA protein–duplex DNA complexes was performed immediately after mixing (within 1 min); perhaps the positively linked affinity effects observed with single-stranded DNA require longer preincubation times with duplex DNA. Additional studies will be required to understand the effects of ATP on duplex DNA binding affinity.

In conclusion, azido-ATP has proven to be a useful ATP analogue for use in recA protein binding studies. The procedure described here is fast and simple and requires very little protein, and thus it is well suited for characterization of the biochemical properties of recA protein, its fragments, or recA mutant proteins. Knight and McEntee (1985a,b) demonstrated the azido-ATP cross-linking can be used to identify the primary amino acid sequence of the ATP binding site within recA protein, and in a complementary application, we demonstrated in Figure 6 that azido-ATP cross-linking can be useful in identifying partial proteolytic digestion fragments of recA protein that are still capable of binding azido-ATP. Isolation and characterization of these fragments (in progress)

[4] Menetski and Kowalczykowski, unpublished observations.

[5] Benedict and Kowalczykowski, submitted for publication.

[6] Kowalczykowski, Clow, and Krupp, submitted for publication.

may lead to the identification of protein fragments that are capable of some partial recA protein reactions. In addition, the azido-ATP analogue should prove useful in quantitative biochemical studies of mutant recA proteins that are defective in ATPase activity, such as recA1 (Ogawa et al., 1978), and such work us currently in progress.

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**Registry No.** ATP, 56-65-5; dATP, 1927-31-7; ATP-$\gamma$-S, 35094-46-3; AMP-PNP, 25612-73-1; UTP, 63-39-8; GTP, 86-01-1; dCTP, 2056-98-6; dGTP, 2564-35-4; ATPase, 9000-83-3; 8-azido-ATP, 53696-59-6.

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