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DNA-strand exchange promoted by RecA protein in the absence of ATP: Implications for the mechanism of energy transduction in protein-promoted nucleic acid transactions

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ABSTRACT **DNA-strand exchange promoted by *Escherichia coli* RecA protein normally requires the presence of ATP and is accompanied by ATP hydrolysis, thereby implying a need for ATP hydrolysis. Previously, ATP hydrolysis was shown not to be required; here we demonstrate furthermore that a nucleoside triphosphate cofactor is not required for DNA-strand exchange. A gratuitous allosteric effector consisting of the noncovalent complex of ADP and aluminum fluoride, ADP·AlF_4^-, can both induce the high-affinity DNA-binding state of RecA protein and support the homologous pairing and exchange of up to 800–900 bp of DNA. These results demonstrate that induction of the functionally active, high-affinity DNA-binding state of RecA protein is needed for RecA protein-promoted DNA-strand exchange and that there is no requirement for a high-energy nucleotide cofactor for the exchange of DNA strands. Consequently, the free energy needed to activate the DNA substrates for DNA-strand exchange is not derived from ATP hydrolysis. Instead, the needed free energy is derived from ligand binding and is transduced to the DNA via the associated ligand-induced structural transitions of the RecA protein–DNA complex; ATP hydrolysis simply destroys the effector ligand. This concept has general applicability to the mechanism of energy transduction by proteins.**

The manner by which enzymes convert the free energy of ATP hydrolysis into useful work is an area of continuing discussion (1–3). Both the controversy and the misperception that surround the molecular mechanisms of energy transduction stem, in part, from the difficulty of measuring precisely both the amount of work accomplished per cycle of ATP hydrolysis and when in the cycle the work itself is accomplished (see ref. 3). This measurement is particularly a challenge for systems where the work is translocation. In this regard, DNA-strand exchange reaction promoted by RecA protein affords an advantage: the work produced by RecA protein is readily measured as the number of base pairs of DNA exchanged between two participating DNA molecules. The RecA protein of *Escherichia coli* can promote the exchange of identical strands of DNA between two DNA homologues, a key step of genetic recombination (4–9). The RecA protein is a DNA-dependent ATPase, and ATP hydrolysis occurs concurrently with DNA-strand exchange. Consequently, it was tacitly assumed that ATP hydrolysis is required for the exchange of DNA strands. Previously, our laboratory (10) and that of Stasiak (11) showed that ATP hydrolysis is not required for the exchange of DNA strands because the nearly nonhydrolyzable analogue, adenosine [γ-thio]triphosphate (ATP[γS]), will support this reaction. This finding casts doubt on the view that ATP hydrolysis is essential for this step of the reaction. We argued that most, if not all, of this hydrolysis is unessential to the work required in the exchange of DNA strands (5, 10, 11). Instead, we advanced the hypothesis that ATP hydrolysis itself does not drive the exchange of DNA strands but, rather, that hydrolysis permits the cycling of RecA protein between two allosteric states: an enzymatically active high-affinity DNA-binding form induced by ATP (or ATP[γS]) binding that is proficient in DNA pairing and exchange versus an inactive low-affinity DNA binding form induced by ADP binding that can rapidly dissociate from DNA (see ref. 5). ATP hydrolysis serves as a molecular switch both to convert RecA protein between active and inactive states and to permit dissociation from DNA and recycling of the protein. Thus, ATP utilization by RecA protein may bear a mechanistic similarity to other energy-transducing proteins such as myosin, EF-Tu, and G proteins (12, 13).

Though there are many examples of systems where NTP binding by an NTPase, but not hydrolysis, is required to enhance binding or self-association reactions (14), few cases exist where net work, as measured by action of the protein on substrates, is achieved in the absence of hydrolysis (see *Discussion*). To demonstrate unequivocally that a work-producing step occurs in the absence of NTP hydrolysis, an absolutely nonhydrolyzable NTP analogue is required or, better yet, an analogue that is not a nucleoside triphosphate. For RecA protein, ATP[γS] is nearly nonhydrolyzable, but, nevertheless, 0.003 molecule of ATP[γS] is hydrolyzed per base pair exchanged (10).

As mentioned above, to unequivocally isolate the work-producing step of the reaction requires an ATP analogue that promotes the requisite allosteric transition, but which itself is not a nucleoside triphosphate. One such potential analogue, the noncovalent complex of ADP and AlF_4^-, inhibits the activity of many ATPases and mimics the ground-state structure of ATP or, more appropriately, ADP·P_i (15–17). Stimulated by the findings that ADP·AlF_4^- activates the co-protease activity of RecA protein (18) and that it induces the structural form of the RecA protein–DNA filament that is observed with ATP or ATP[γS] (19), we examined directly whether ADP·AlF_4^- can induce the high-affinity DNA-binding state of RecA protein and whether it can support the DNA-strand exchange reaction. We find that ADP·AlF_4^- is, indeed, a suitable effector molecule despite the absence of a high-energy phosphodiester bond and that activation of RecA protein, homologous DNA sequence recognition, and exchange of DNA strands can occur in the complete absence of a nucleoside triphosphate. The significance of these results is discussed in the context of both DNA-strand exchange processes and the mechanism of energy transduction by other NTPases, both nucleic acid-dependent and -independent.

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Abbreviations: ATP[γS], adenosine [γ-thio]triphosphate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RFI, relative fluorescence increase.

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EXPERIMENTAL PROCEDURES

Reagents. All chemicals were reagent grade, and solutions were made in glass-distilled H_2O. ADP was purchased from a variety of vendors (Boehringer Mannheim, Pharmacia, and Sigma) and dissolved as a concentrated stock at pH 7.5. ADP stocks were assayed for the presence of contaminating ATP by the bioluminescent luciferase assay kit supplied by Sigma (technical bulletin no. BAAB-1). The ADP (Sigma) found most free of ATP contamination was further purified by ion-exchange chromatography on a preparative (10 × 125 mm) Nucleogen (Macherey-Nagel, Germany) DEAE 4000-10 HPLC column and used immediately in enzymatic assays. No differences with regard to RecA protein-dependent behavior were observed for any of these ADP preparations. NaF and $Al(NO_3)_3$ were obtained from J. T. Baker Chemical and EM Science, respectively. Proteins and DNA were purified and quantified as described (10).

DNA-Binding Assay. DNA binding was measured as described (20). The reaction buffer consisted of 20 mM Tris acetate, pH 7.5/0.1 mM dithiothreitol/10 mM $Mg(C_2H_3O_2)_2$/0.5 mM ADP/10 mM NaF/40 μM $Al(NO_3)_3$. Complexes were formed using 1.2 μM RecA protein and 3 μM etheno-M13 single-stranded DNA (ssDNA) at 37°C and allowed to equilibrate until the fluorescence signal was stable; then aliquots of 5 M NaCl were added to dissociate the complexes. The relative fluorescence increase (RFI) due to RecA protein–etheno-DNA complex formation was measured as described (20).

DNA-Strand Exchange Assay. DNA-strand exchange was measured as described (10). The reaction buffer consisted of 25 mM Tris acetate, pH 7.5/1 mM dithiothreitol/10 mM $Mg(C_2H_3O_2)_2$/5 mM ADP/10 mM NaF/0.4 mM $Al(NO_3)_3$. Presynaptic complexes were formed by preincubating 10 μM M13mp7 ssDNA with 0.9 μM ssDNA-binding protein for 10 min at 37°C, followed by the addition of 6 μM RecA protein and the ADP, NaF, and $Al(NO_3)_3$. After 10 min, 20 μM M13mp7 double-stranded DNA (dsDNA) that was linearized with *Eco*RI endonuclease was added to initiate the reaction. Although the order of protein addition was not crucial, the omission of ssDNA-binding protein reduced the yield of joint molecules to <4% (data not shown). Aliquots of the reaction were stopped by the addition of 1% SDS/50 mM EDTA and incubated at 37°C for 10 min; they were loaded on an 0.8% agarose gel that was run in the absence of ethidium bromide and then stained.

S1 Nuclease Assay. DNA heteroduplex formation was measured using the S1 nuclease assay (21) exactly as described (10). Reaction conditions were identical to those described for the DNA-strand exchange assays, except that the M13mp7 linear dsDNA was tritium-labeled. Joint molecule formation was determined on a portion of the assay mixture by using the DNA-strand-exchange assay and quantified using a Zeineh laser densitometer. The average size of the DNA heteroduplex region per joint molecule was calculated as described (10).

RESULTS

ADP·AlF_4^- Induces the High-Affinity DNA-Binding State of RecA Protein. Fig. 1 shows the results from a DNA-binding assay used to detect induction of the high-affinity state of RecA protein (20). This assay employs a chemically modified fluorescent M13 ssDNA, referred to as etheno-M13 DNA. The binding of RecA protein to etheno-M13 DNA results in a protein–DNA complex whose characteristics depend on the nucleotide cofactor present. The active, high-affinity DNA-binding state of RecA protein induced by ATP is characterized by a ternary complex that both has a higher RFI [indicative of an extended filamentous structure (5)] and requires a greater NaCl concentration for dissociation. Fig. 1 shows that ADP·AlF_4^- can elicit the behavior typical of the high-affinity

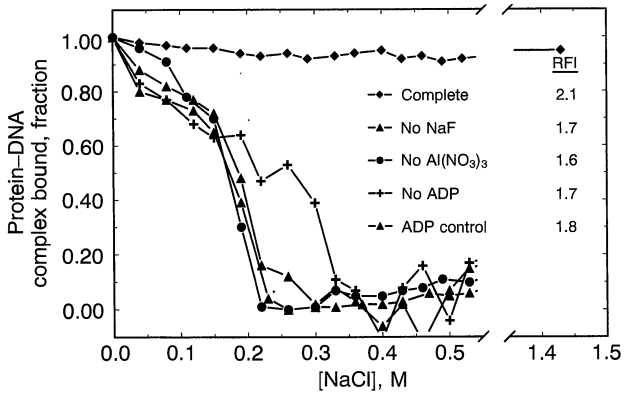

FIG. 1. Induction of the high-affinity DNA-binding state of RecA protein by ADP·AlF_4^-. DNA-binding assays were conducted as described in *Experimental Procedures*. For the ADP control, NaF and $Al(NO_3)_3$ were omitted. The increased scatter in the No ADP data results from a slight turbidity arising from the aggregation of RecA protein that occurs in the absence of nucleotide cofactors.

state; neither ADP, $Al(NO_3)_3$, nor NaF alone, or in any pair-wise combination, can induce the high-affinity state. Formation of the high-affinity DNA-binding state is still detected when the reagent concentrations are lowered individually to concentrations as low as 1 mM $Mg(C_2H_3O_2)_2$, 25 μM ADP, 1.5 mM NaF, or 0.1 $Al(NO_3)_3$ but is not detected below 10 μM ADP or 0.5 mM NaF (data not shown). The RecA protein–DNA complex formed with ADP·AlF_4^- resembles the complex formed with ATP[γS] in the following ways: it is stable to >1 M NaCl; it yields a protein–DNA complex with a similar RFI; it forms a complex whose fluorescence signal saturates at a stoichiometry of one RecA protein monomer per 6–7 nucleotide residues (data not shown; ref. 22); and it has a nearly identical filamentous structure (19). Thus, ADP·AlF_4^- mimics the behavior of both ATP and ATP[γS] and is, consequently, an allosteric inducer of the active state of RecA protein.

ADP·AlF_4^- Supports RecA Protein-Promoted DNA-Strand Exchange. Because induction of the high-affinity state of RecA protein is a necessary, although not always sufficient, condition for DNA-strand exchange activity (5), the ability of RecA protein to homologously pair and exchange strands between M13 ssDNA and homologous linear dsDNA was examined (Fig. 2). The reaction with ATP displays the typical temporal appearance of joint molecule intermediates followed by conversion to the product, gapped circular dsDNA. The reaction with ADP·AlF_4^- demonstrates that homologously paired joint molecules can form; however, as for the ATP[γS]-dependent reaction, no gapped dsDNA is detected, indicating that extension of the DNA heteroduplex region is blocked (10).

Joint molecule formation in the ADP·AlF_4^--dependent reaction displays distinctive reaction requirements, being optimal at 5 mM ADP/0.4 mM $Al(NO_3)_3$/10 mM NaF/10 mM $Mg(C_2H_3O_2)_2$; the use of reagent concentrations that are either higher or lower than these optima results in a reduced final yield of joint molecules (Fig. 3). In agreement with the DNA-binding experiments, the omission of any reagent prevents joint molecule formation. Finally, as observed for ATP-dependent joint molecule formation, joint molecule formation is sigmoid in RecA protein concentration (no joint molecule formation occurs at 0.5 μM RecA protein), and optimal pairing activity requires approximately one RecA protein monomer per 3 nucleotides of ssDNA (data not shown). Finally, pairing does not occur when heterologous pBR322 dsDNA is substituted for the M13 dsDNA (data not shown).

The length of the region of DNA heteroduplex present in the joint molecules can be determined by means of an S1 nuclease assay (21). The time courses for both joint molecule and DNA

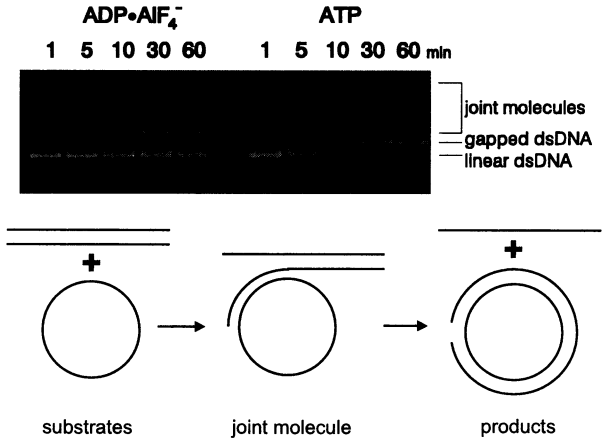

FIG. 2. DNA-strand exchange promoted by RecA protein in the presence of ADP·AlF_4^-. The positions of the linear dsDNA substrate, joint molecules intermediate, and gapped dsDNA product molecules are indicated. The minutes shown represent the time of reaction after addition of linear dsDNA. For comparison, a reaction with ATP [but without ADP, NaF, and $Al(NO_3)_3$] under otherwise identical conditions is shown.

heteroduplex formation are shown in Fig. 4. Approximately 800–900 bp of heteroduplex DNA are formed per joint molecule, and the size of the heteroduplex joint remains invariant after ≈10 min; for comparison, the ATP[γS]-dependent reaction resulted in joint molecules containing slightly more than 3000 bp of heteroduplex DNA after ≈10 min (10). The reason for the different extents of branch migration for the ADP·AlF_4^- and ATP[γS] reactions is unclear but may be due to an increased number of discontinuities in presynaptic filaments formed with ADP·AlF_4^- (10).

DNA-Strand Exchange in the Presence of ADP·AlF_4^- Is Not Due to ATP Contamination. The conclusion that DNA-strand exchange is occurring in the absence of ATP obviously would be compromised by the presence of contaminating ATP in any of the reagents. However, a number of control experiments (data not shown) argue against this possibility. (*i*) Joint molecule formation was independent of the source of ADP (three different commercial sources); luciferase assays indicated the presence of ATP at molar ratios ranging from 7:100,000 to 3:10,000, depending on the source. (*ii*) Purification of the cleanest ADP to reduce the ATP content to <0.7 ppm did not change the results (at 5 mM ADP, the ATP contamination was <3.5 nM). (*iii*) Increasing the ADP concentration to 25 mM completely inhibited homologous pairing activity (Fig. 3), inconsistent with the notion that contaminating ATP is responsible for pairing activity. (*iv*) There was no detectable ATP (<0.1 μM) in either the inorganic reagents, the RecA or ssDNA-binding proteins (assayed in both their native and denatured forms), or in the DNA when present at the concentrations indicated in Fig. 2. (*v*) Addition of 10 μM ATP to reaction mixtures containing ADP·AlF_4^- did not alter the reaction profile. Thus, the observed RecA protein-promoted DNA-strand exchange activity in the presence of ADP·AlF_4^- cannot be attributed to contamination by ATP.

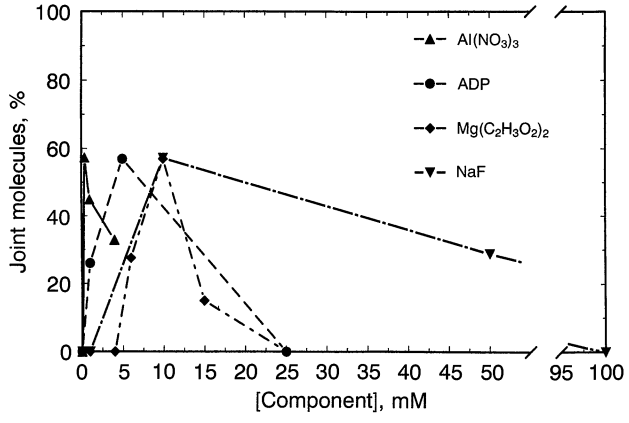

FIG. 3. Effect of reagent concentrations on the final yield of joint molecules formed in the presence of ADP·AlF_4^-. Reactions such as those shown in Fig. 2 were monitored over a 60-min time course; in all cases, the final reaction endpoint was achieved in 30 min or less. Reaction conditions were those described in *Experimental Procedures*, except that the concentration of the indicated component was varied. Gels were quantified by densitometric scanning of the negative, and the final yield of joint molecules is presented.

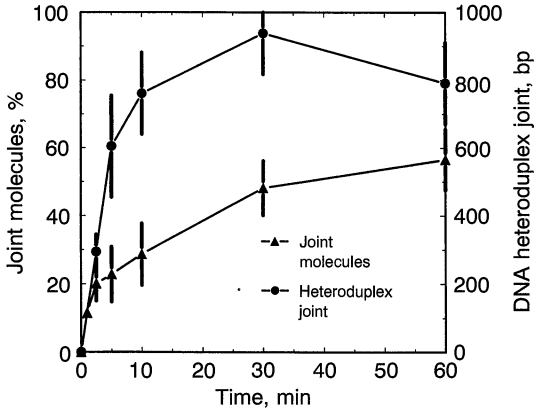

FIG. 4. Extent of RecA protein-promoted DNA heteroduplex formation in the presence of ADP·AlF_4^-. The percentage of dsDNA converted into joint molecules was determined for a portion of the assay mixture using the agarose gel assay and is plotted as a function of time (▲). The amount of DNA heteroduplex formed was determined using the S1 nuclease assay, and average size of the DNA heteroduplex region per joint molecule formed is plotted (●).

DISCUSSION

These results demonstrate conclusively that free energy derived from ATP hydrolysis is not essential for DNA-strand exchange (10). Instead, the free energy derived from the binding of an appropriate nucleotide cofactor is sufficient to promote DNA-strand exchange by RecA protein. How does this occur? As we have argued elsewhere, the allosteric transition induced by ATP (or dATP, ATP[γS], or ADP·AlF_4^-) binding results in a protein that can stabilize a transition state essential to DNA-strand exchange (Fig. 5, refs. 5 and 10). We suggested that this intermediate contains three strands of DNA paired in such a way that they are poised for the final exchange of DNA strands and that, due to the energy of activation required for formation, it cannot form in the absence of RecA protein (5, 10). This intermediate must closely resemble the final products of DNA-stand exchange because product molecules with fully exchanged DNA strands are found when shorter DNA substrates are used (5, 11) and because analysis of DNA-strand disposition in the RecA protein–DNA-strand exchange complex by chemical means is consistent with a product-like structure (23).

Though net DNA strand exchange is isoenergetic, formation of a homologously paired DNA-strand-exchange intermediate requires overcoming a considerable activation energy barrier (see ref. 9): (*i*) the act of bringing two DNA molecules sufficiently close to one another to permit homologous recognition through non-Watson–Crick hydrogen bonding results in a three-stranded DNA structure with a higher charge density than that of dsDNA, imposing an associated electrostatic energetic cost; (*ii*) DNA-strand exchange also entails the energetic cost required to disrupt the preexisting base-pairing

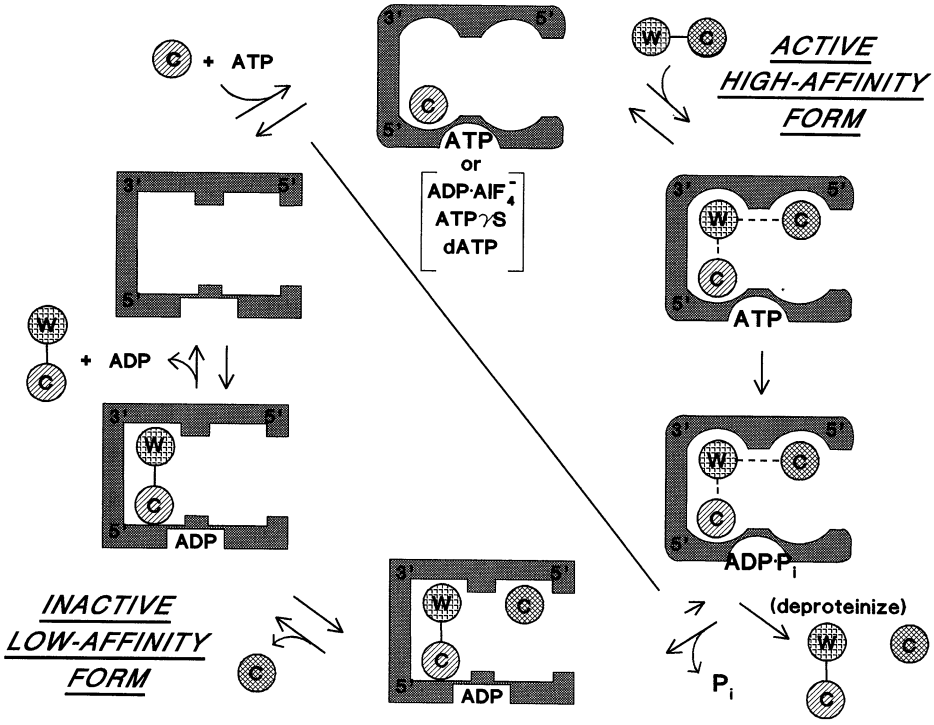

FIG. 5. Schematic model for DNA-strand exchange promoted by RecA protein in the absence of ATP or ATP hydrolysis [adapted and reprinted with permission from ref. 5 (copyright Annual Reviews Inc., Palo Alto, CA, 1991)]. Hatched C, ssDNA strand (Crick) within presynaptic filament; crosshatched C, identical ssDNA strand (Crick) of DNA duplex; W, complementary ssDNA strand (Watson) of DNA duplex.

and base-stacking of the substrate dsDNA. Both of these energetic costs required to form the intermediate are provided by the free energy derived from the binding of ATP to RecA protein. ATP hydrolysis serves simply to produce ADP, which, in turn, destabilizes the protein–DNA complex, permitting net exchange and release of DNA strands from the protein. Though ATP hydrolysis is not needed for the physical step of DNA-strand exchange, it nevertheless is important to the overall DNA-strand exchange reaction (i.e., cycle). As elaborated elsewhere (5), ATP hydrolysis does serve several important functions, but these functions relate primarily to kinetic aspects of DNA-strand exchange: it is required for the formation of a contiguous filament of RecA protein bound to ssDNA via continued dissociation and reassembly of RecA protein protomers; it is needed for DNA heteroduplex extension for similar reasons; it is needed to bypass heterologous blocks encountered during DNA-strand exchange by an unknown kinetic mechanism (24, 25); and, finally, it imparts directionality to the process (11).

The lack of a requirement for ATP hydrolysis and, now, for even a nucleoside triphosphate cofactor seems to also reconcile the finding that DNA-strand-exchange proteins isolated from eukaryotic sources appear to function without the need for exogenously added nucleoside triphosphate (for reviews, see refs. 9 and 26). However, the discovery that these proteins either possess or require an associated nuclease activity argues that they function by a different mechanism (9). Homologous pairing by these proteins requires exonucleolytic resection of the linear dsDNA substrate and is followed by reannealing of this ssDNA tail with the other ssDNA substrate; hence, these proteins do not initiate DNA-strand exchange within a region of intact dsDNA. Because DNA reannealing is an energetically favorable process, the need for ATP is averted; in agreement, pairing and spontaneous branch migration between ssDNA and resected dsDNA can be mediated by DNA condensing agents, in the absence of pairing proteins (27, 28). Although the mechanism by which these proteins accelerate the renaturation process remains an interesting issue, it appears that they function by a mechanism distinct from that used by proteins such as RecA protein. However, recently it was found that one of these protein, SEP1 of *Saccharomyces cerevisiae*, can promote paranemic pairing between dsDNA and ssDNA (29). Even though the mechanism of this latter reaction is unknown, it nevertheless is likely that the general energetic considerations discussed above are universally applicable: proteins that are capable of binding two different DNA molecules, shielding the repulsive electrostatic charges, and distorting dsDNA structure can, in principle, promote homologous pairing and DNA-strand exchange without ATP binding or hydrolysis; however, turnover of protein from the product complexes will be rate-limiting.

Nucleoside triphosphatases are involved in nearly all biological processes that are energetically unfavorable, and nucleic acid-dependent NTPases are commonplace. However, the specific mechanistic role of NTP hydrolysis in most of these processes is unknown. Ligand-induced changes in protein structure are a common feature of these ATPases and GTPases (30–34), and the mechanism used by RecA protein to convert the free energy of a chemical step into mechanical work is thermodynamically similar to that invoked for a number of energy-transducing systems [e.g., myosin (12), dynein (35), membrane ATPases (36), chaperon proteins (37–39), kinesin (40), and the GTPases: EF-Tu, G proteins, signal recognition particle receptor, and tubulin (13, 30–32, 34, 41, 42)]. Each of these proteins can exist in either of two conformations: active vs. inactive or high affinity vs. low affinity. Although these systems differ with regard to nature of the specific liganded state that elicits the high-affinity substrate-binding state (see, e.g., refs. 2 and 40), this general mechanism—i.e., the use of ATP hydrolysis to change the state of ligand occupancy so as to switch between two functionally nonequivalent conformers of an enzyme—is a general theme in energy transduction. This principle is also applicable to other ATP-dependent enzymes that act on nucleic acids, such as DNA gyrase (43, 44), DnaA protein (45), Mu B protein (46, 47), and DNA helicases (48). For DnaA protein, the ATP-

bound form is the active form, whereas the ADP-bound form is inactive. Similarly for Mu B protein, the ATP-bound form displays high DNA-binding affinity, whereas the ADP-bound form dissociates rapidly from DNA. Comparable functional interconversions may occur when DNA helicases act to convert dsDNA to ssDNA.

For enzymes that couple the hydrolysis of NTP to another reaction, the work- or power-producing step in the reaction cycle is, in most cases, unknown. However, there are two examples where the use of ligand-binding free energy parallels that of RecA protein where the coupled enzymatic work occurs before ATP hydrolysis. Both *Escherichia coli* DNA gyrase (43) and *Drosophila melanogaster* topoisomerase II (49) normally require ATP hydrolysis to introduce supercoiling in DNA. However, both are capable of inducing changes in DNA supercoiling when the apparently nonhydrolyzable ATP analogue AMPPNP (5′-adenylyl-β,γ-imidodiphosphate) is used. In the presence of AMPPNP, negative supercoiling occurs, but the reaction is not catalytic. Instead, the extent of supercoiling depends on the enzyme concentration because, although enzymatic action (i.e., DNA-strand breakage and passage) is supported by AMPPNP, release of the enzyme from the DNA does not occur; in the case of *D. melanogaster* topoisomerase II, a high-affinity noncovalent enzyme–DNA complex persists (49). Thus, like for RecA protein, the free energy needed for enzymatic work, in this case supercoiling, is derived from ATP binding to the protein; ATP hydrolysis is needed only for turnover. Consequently, for proteins such as RecA protein and type II topoisomerases, the free energy of NTP hydrolysis is used to permit and to regulate interconversion between these different functional forms but is not used to do enzymatic work directly; the work requires only NTP binding and is completed before hydrolysis occurs.

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