Evidence for Nucleotide-Mediated Changes in the Domain Structure of the *Rec*A Protein of *Escherichia coli*†

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ABSTRACT: We have used limited trypsin digestion as a means of investigating changes in the structural properties of *rec*A protein accompanying the binding of different nucleoside triphosphates. The levels of four partial digestion products are greatly increased in digests of *rec*A protein complexed with dTTP, dATP, ATP, or the ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS). These bands (22, 19, and 17.5 kilodaltons) are absent or present at reduced levels in digests of *rec*A protein alone. Unlike these nucleotides, all of which bind tightly to *rec*A protein, nucleotides and analogues that bind poorly produce little or no change in the digestion pattern of *rec*A protein. We have compared the rates of fragment accumulation in the presence of dTTP and show a saturable dependence on nucleotide concentration. Binding of single-stranded DNA to *rec*A protein does not alter the pattern of digestion products compared to protein alone, and the digestion pattern of *rec*A protein–DNA–ATPγS ternary complexes is similar to that of uncomplexed enzyme. We have used monoclonal antibody binding, high-performance liquid chromatography separation of peptides, and amino acid composition analyses to localize the regions of *rec*A protein which are altered in their susceptibility to trypsin when nucleoside triphosphates are present. The results of these analyses indicate that the fragments arise from trypsin cutting at two or more sites near the middle of the primary sequence. These cleavage sites are more than 80–110 residues away from the site of photoaffinity labeling by 8-N_3ATP (Tyr-264). Our results suggest that, in the presence of certain nucleotides, *rec*A protein is organized into two stable structural domains.

The *rec*A protein of *Escherichia coli* is an unusually versatile enzyme. This 38 000-dalton protein catalyzes or promotes several reactions including (i) DNA-dependent hydrolysis of certain nucleoside triphosphates (NTP's)[1] (Ogawa et al., 1978; Weinstock et al., 1981a); (ii) ATP-dependent transfer of single-stranded DNA into homologous duplexes (strand transfer or assimilation) (Shibata et al., 1981; McEntee et al., 1980) as well as renaturation of complementary single-stranded DNA chains (Weinstock et al., 1979; McEntee, 1985); and (iii) polynucleotide and nucleoside triphosphate dependent cleavage of phage λ repressor (cI protein) (Craig & Roberts, 1980; Roberts et al., 1978) and the cellular LexA repressor which regulates expression of more than 15 DNA damage inducible genes (SOS genes) (Little et al., 1980; Horii et al., 1981; Walker, 1984). Biochemical characterization of these reactions has demonstrated a requirement for nucleoside triphosphate binding but not hydrolysis in processes ii and iii. Thus, ATP and certain other nucleoside triphosphates can serve as either substrates or effectors of *rec*A protein activities.

Biochemical and kinetic studies (Weinstock et al., 1981c; Knight & McEntee, 1985a; Cotterill et al., 1982) are consistent with a model in which each monomer of *rec*A protein contains a single binding site for nucleoside triphosphates. Recently, we have demonstrated that the ATP photoaffinity analogue 8-N_3ATP covalently modifies a single tyrosine residue (Tyr-264) in *rec*A protein which is also the site of modification by 5′-FSBA (Knight & McEntee, 1985b). The photolabeling of this tyrosine residue is competed efficiently by nucleoside triphosphates and analogues that competitively inhibit ATP hydrolysis (Knight & McEntee, 1985c). Additional evidence that Tyr-264 is located within the ATP binding domain of *rec*A protein has come from experiments in which an isolated 24-residue tryptic fragment (T31) containing residues 257–280 of *rec*A protein was photoaffinity labeled with high efficiency and specificity (Knight & McEntee, 1986). These latter results further support the idea that an important portion of the ATP binding domain of *rec*A protein is localized within a relatively small region of the primary structure of the enzyme.

As part of our analysis of the interaction of *rec*A protein with ATP and other nucleoside triphosphates, we have performed limited protease digestions of *rec*A protein and *rec*A protein–NTP complexes. Analysis of the tryptic digestion products by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) demonstrates that four bands which are present in digests of *rec*A protein–NTP complexes are absent or present at considerably lower levels in digests of uncomplexed protein. Using a combination of HPLC, amino acid composition determination, and monoclonal antibody binding, we have found that these digestion products result from two or more trypsin cuts located approximately in the middle of the primary sequence. These results are consistent with an allosteric role for nucleoside triphosphates which induce or stabilize a conformation of *rec*A protein containing two distinct structural domains.

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[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); NTP, nucleoside triphosphate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; kb, kilobase(s); bp, base pair(s); SSB, single-stranded binding; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 5′-FSBA, 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine.

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Experimental Procedures

Materials

Enzymes. *Rec*A protein was purified from *E. coli* K12 strain KM1842 as described (Cox et al., 1981). The enzyme was greater than 98% homogeneous as judged by densitometry of silver-stained polyacryamide gels containing 5 μg of protein. The *rec*A protein was stored at −70 °C in R buffer [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 1 mM dithiothreitol] containing 25% glycerol. The concentration of *rec*A protein was determined by using a value of $\epsilon_{280} = 5.16$ (Weinstock et al., 1981a). The *E. coli* helix destabilizing protein was generously provided by Dr. Robert Fuller, Stanford University.

Trypsin (Worthington) solutions (0.1 mg/mL in 0.1 mM HCl) were prepared immediately before use. Nucleoside triphosphates, diphosphates, and nucleotides were purchased from Sigma; ATPγS and AMPPNP were purchased from Boehringer Mannheim.

Antibodies. Monoclonal antibody prepared against *rec*A protein (Ab156) was obtained from Dr. Alex Karu, Naval Biosciences Laboratory, University of California, Berkeley, CA. Rabbit antibodies were raised against *rec*A protein as described (Keener et al., 1984).

Plasmids. Strains of *E. coli* containing *rec*A–*lac*Z protein fusions were obtained from Dr. George Weinstock, The University of Texas, Houston, TX. Plasmid pGE245 contains the same *rec*A–*lac*Z fusion as plasmid pGE113 (Weisemann et al., 1984) consisting of the first 47 codons of the *rec*A coding sequence fused in frame to 1015 residues of β-galactosidase. Plasmid pGE271 contains the first 308 residues of *rec*A protein fused to β-galactosidase (Bremer et al., 1984). Both plasmids contain upstream operator sequences necessary for *Lex*A protein regulation of these fusion products. The truncated *rec*A gene was prepared by digesting a 3.0 kb *Bam*HI fragment containing the complete *rec*A gene and its control region with *Eco*RI. The proximal 1.77 kb *Bam*HI–*Eco*RI fragment containing the regulatory region and the first 777 bp of coding sequence was cloned into *Bam*HI–*Eco*RI-digested pBR322. Plasmid pJC917 encodes a protein consisting of the first 6 residues of β-galactosidase fused in frame to the carboxyl-terminal 92 residues of *rec*A protein (A. J. Clark, personal communication).

Methods

Limited Trypsin Digestion of RecA Protein. Typical reactions mixtures contained 100 μg of *rec*A protein in a volume of 100 μL. *Rec*A protein was incubated with or without nucleoside triphosphate in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM KCl for 20 min at 37 °C. Trypsin was added to a final enzyme to substrate ratio of 1:200 (w/w) in most experiments, but for shorter digestions, a ratio of 1:100 was used. Trypsin digestions were performed at 25 °C. At the indicated time after trypsin addition, aliquots (8 μg) were added to Laemmli sample buffer [62.5 mM Tris (pH 6.8), 10% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue], heated to 100 °C for 2 min to stop the digestion, and loaded onto polyacrylamide gels containing SDS. Following electrophoresis, gels were stained by using either Coomassie Brilliant Blue or silver nitrate as described (Merril et al., 1981), or they were transferred to nitrocellulose without staining for Western blot analysis (see below). Densitometry of silver-stained polyacrylamide gels was performed by using a Helena Quikscan Junior densitometer (Helena Laboratories).

Preparation of Partial Tryptic Fragments of RecA Protein. Fragments of *rec*A protein resulting from limited digestion with trypsin were purified as follows. After completion of the limited trypsin digest, 50 μL of glycerol, 50 μL of 10% SDS, and 300 μL of Laemmli sample buffer were added to 1.0 mL of reaction mixture. The entire volume was loaded onto a preparative SDS–polyacrylamide gel [15% acrylamide/0.95% bis(acrylamide)]. Following electrophoresis, gels were stained briefly with Coomassie Brilliant Blue. Bands of interest were cut out of the gel, and protein was extracted by electroelution into buffer containing 10 mM NH_4HCO_3 and 0.02% SDS as described (Hunkapillar et al., 1983). Yields were estimated by analytical SDS–polyacrylamide gel electrophoresis using known amounts of single-stranded binding (SSB) protein from *Escherichia coli* as standards. Although the fragments isolated by this procedure were extremely pure on the basis of SDS–polyacrylamide gel analysis and HPLC examination, in several attempts we were unable to dansylate or to sequence the gel-isolated fragments using procedures that we had used successfully for analyzing tryptic fragments isolated using two-dimensional peptide mapping techniques (Knight et al., 1984).

Complete Trypsin Digestion of Gel-Purified Peptides. The isolated fragments were prepared for complete proteolytic digestion by extraction of the Coomassie and SDS using solvent system A (acetone/triethylamine/acetic acid/water, 17:1:1:1) as described (Konigsberg & Henderson, 1983). Precipitated protein fragments were solubilized in 50 μL of 6 M urea and diluted 3-fold with 0.1 M NH_4HCO_3. $CaCl_2$ was added to a final concentration of 0.1 mM, and trypsin was added to a final enzyme to substrate ratio of 1:50 (w/w). Following incubation at 37 °C for 12 h, digestions were either stopped by quick freezing in a dry ice/ethanol bath or loaded directly onto HPLC for purification of resulting peptides.

HPLC Purification and Amino Acid Composition Analysis of Tryptic Peptides. HPLC purification of peptides was performed with Perkin-Elmer reverse-phase cartridge columns (Peco-sphere C-18, 4.6 mm × 150 mm, 5-μm particle size). Samples were loaded onto columns equilibrated in solvent A (20 mM sodium phosphate, pH 6.8). Profiles were developed with a linear gradient of solvent B (70% CH_3CN) into solvent A, 0–84 min (0–60% solvent B), at a flow rate of 1.0 mL/min. The column eluents were monitored at 229 and 280 nm and fractions collected every minute. Peptides that eluted as a clustered set of peaks were separated by a second C-18 HPLC step using shallow gradient conditions as follows. Appropriate fractions were pooled, lyophilized, and dissolved in solvent A containing 4 M guanidine hydrochloride. Profiles were developed as described above except that the rate of introduction of solvent B into solvent A was decreased approximately 5-fold in the region where peptide elution occurred. Fractions containing peptide peaks were pooled separately and lyophilized. Amino acid analysis was performed as described (Knight & McEntee, 1985a).

Western Blotting Analysis of RecA Tryptic Fragments and Fusion Proteins. The procedure employed for Western blot analysis of *rec*A protein fragments and fusion proteins has been described (Keener et al., 1984). Crude cell extracts were prepared by a small-scale version of the cell lysis and extract preparation procedures described (Cox et al., 1981).

Results

Nucleoside Triphosphates Alter the Pattern of Tryptic Digestion Products of RecA Protein. Incubation of *rec*A protein with low concentrations of trypsin (1:200 w/w) resulted in the appearance of a series of relatively large peptides that could be resolved by electrophoresis in an SDS–polyacrylamide gel (Figure 1, −NTP lanes). Under the conditions used in this

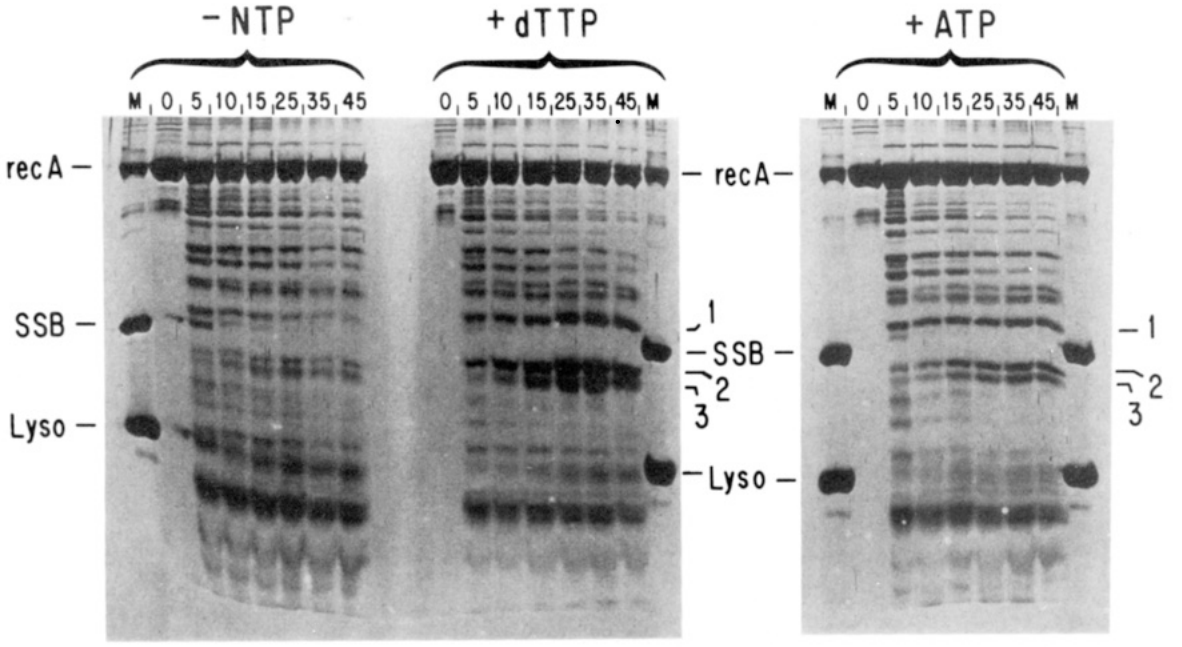

FIGURE 1: Binding of dTTP or ATP alters the pattern of trypsin cleavage of *rec*A protein. Purified *rec*A protein was incubated with trypsin [enzyme:substrate ratio of 1:200 (w/w)] at 25 °C for the time (minutes) indicated above each lane. Aliquots containing 8 μg of *rec*A protein were mixed with gel loading buffer heated to 100 °C for 2 min to terminate the cleavage reaction and analyzed by electrophoresis in SDS–polyacrylamide gels (12% acrylamide, 0.76% cross-linking). The digests were visualized by silver staining. −NTP, *rec*A protein alone; +dTTP, *rec*A protein plus 1 mM dTTP; +ATP, *rec*A protein plus 1 mM ATP; M, marker lane containing *rec*A protein (37.8K), *E. coli* helix destabilizing protein (SSB) (19K), and egg white lysozyme (Lyso) (14.4K). Fragments 1, 2, and 3 are partial tryptic digestion products that accumulate when *rec*A protein is complexed with dTTP or ATP.

experiment, approximately half of the *rec*A protein in the reaction was digested after 45 min as judged by the reduction in the amount of intact *rec*A protein. Changes in the relative intensities of partial digestion products could still be observed after 40 min of incubation, indicating that the trypsin was active throughout the digestion period.

A change in the protein digestion pattern was observed when *rec*A protein was complexed with certain nucleoside triphosphates. In the presence of ATP (1 mM), three bands accumulated in the tryptic digests that were produced at significantly lower levels in the absence of ATP (Figure 1, +ATP lanes), bands 1, 2, and 3. These bands were generated within the first minutes of treatment, and their levels increased during the first 30 min of digestion. Incubation of *rec*A protein with dTTP caused an even greater increase in the amount of these three digestion products (Figure 1, +dTTP lanes). On the basis of their relative mobilities in SDS–polyacrylamide gels, we estimate the molecular weights of bands 1, 2, and 3 to be approximately 22K, 19K, and 17.5K, respectively.

The digestion patterns of *rec*A protein alone or complexed with ligand were reproducible with respect to both the sizes and relative amounts of the partial tryptic peptides produced. In some experiments, we observed slight changes in the intensity of one or two large fragments, particularly at early times during digestion, but we did not investigate these effects further.

We examined the effects of several other nucleoside triphosphates, diphosphates, and analogues on the partial tryptic digestion pattern of *rec*A protein in order to determine the specificity of this effect. As shown in Figure 2, when dATP, ADP, or ATPγS was included in the incubation with trypsin and *rec*A protein, bands 1–3 accumulated to high levels. However, when the ATP analogue AMPPNP was included in the incubation, only slight changes were detected in the pattern of tryptic products when compared to a digestion of *rec*A protein alone (Figure 2). In a similar set of experiments, we observed that UTP (1 mM) stimulated production of bands 1–3 in tryptic digests of *rec*A protein whereas addition of adenosine (1 mM) resulted in a digestion pattern that was identical with *rec*A protein alone (data not shown).

We performed a series of control experiments to determine whether the changes in digestion products were due to the effect of nucleoside triphosphates on trypsin rather than *rec*A protein. We found that inclusion of dTTP (1 mM) during trypsin digestion of bovine serum albumin did not alter the kinetics or products of proteolysis (data not shown). Moreover, we could demonstrate that the appearance of bands 1–3 required a native *rec*A protein structure since no increase in their intensities was seen when heat-inactivated *rec*A protein was incubated with dTTP (data not shown). In an additional experiment, we found that Mg^{2+} was not required for the dTTP-dependent accumulation of these partial digestion products (data not shown).

We compared the relative intensity of band 1 after 35 min of incubation for reactions containing different nucleoside triphosphates, diphosphates, and analogues in order to determine the relative efficacy of these ligands for altering the pattern of tryptic digestion products. The relative effectiveness was found to be dTTP, dATP > ATPγS, ADP > ATP, UTP. Moreover, AMPPNP and adenosine addition produced no enhancement of band 1 (Figure 2; data not shown). Some of these nucleotides and analogues have been shown to bind to *rec*A protein and either be hydrolyzed in the presence of DNA (dATP, ATP, UTP) or competitively inhibit ATP hydrolysis (dTTP, ADP, ATPγS) (Weinstock et al., 1981b). Competition experiments demonstrate that neither AMPPNP nor adenosine binds tightly to *rec*A protein (Lovett & Roberts, 1985; Knight & McEntee, 1985a).

Kinetics of Accumulation in the Presence of ATP or dTTP. The intensities of bands 1–3 were determined by densitometry of silver-stained gels at different times after addition of trypsin (Figure 3). In the absence of nucleoside triphosphates, the amounts of protein migrating at the positions of bands 1–3 remained low and relatively constant throughout the course

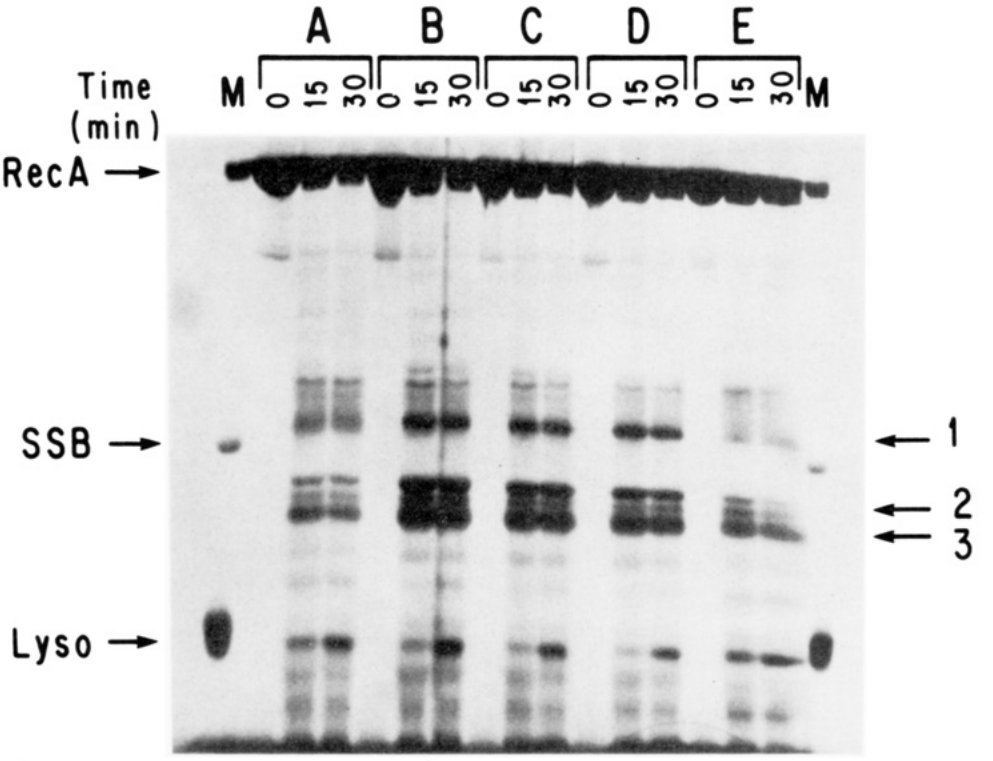

FIGURE 2: Effects of other nucleotides and analogues on trypsin cleavage of *rec*A protein. *Rec*A protein was incubated with the indicated nucleotide or analogue (1 mM final concentration), and trypsin was added to a final enzyme:substrate ratio of 1:100 (w/w). Digestion was terminated after 15 or 30 min by addition of gel loading buffer and heating to 100 °C for 2 min. Fragments were separated by electrophoresis in a SDS–polyacrylamide gel (12% acrylamide, 0.76% cross-link). The fragments were visualized by silver staining. The band migrating between bands 2 and 3 was not reproducibly detected and probably results from incomplete denaturation of the sample. Each lane contained 8 μg of *rec*A protein. M, marker lanes containing *rec*A protein, *E. coli* SSB protein, and lysozyme. (A) −NTP, no nucleotide; (B) +1 mM dATP; (C) +1 mM ADP; (D) +1 mM ATPγS; (E) +1 mM AMPPNP.

of digestion. In the presence of ATP or dTTP, the levels of bands 1–3 increased steadily for more than 30 min of digestion after which the amounts of these products decreased, presumably due to further trypsin cleavage. The maximal levels of fragments were 2–4 times higher when *rec*A protein was incubated with dTTP compared to ATP. In the case of dTTP, band 2 accumulates most rapidly during the first 20 min of digestion, and bands 1 and 3 display slower kinetics. In the presence of either ATP or dTTP, band 3 levels lag for approximately the first 10 min and then show a steady rise.

dTTP Concentration Dependence of Trypsin Cleavage. We investigated the levels of fragments 2 and 3 at several different concentrations of dTTP after 20 min of trypsin digestion. The band intensities were determined by densitometry of silver-stained gels, and the results of this experiment are shown in Figure 4. The levels of bands 2 and 3 increase rapidly below 50 μM dTTP, and at higher ligand concentrations, a saturable dependence is observed. Thus, the increase in the intensities of bands 2 and 3 occurs in a concentration range of ligand (10–70 μM) which is comparable to the dTTP concentrations that inhibit ATP hydrolysis by *rec*A protein (K_I = 20 μM) (Weinstock et al., 1981b). The saturation level of band 2 was approximately twice that of band 3 after 20 min of digestion, as judged by densitometry of the stained gel.

Localization of Nucleotide-Induced Tryptic Fragments. As a first step toward determining the regions of *recA* protein which showed altered accessibility to trypsin cleavage in the presence of nucleotides, we incubated *rec*A protein with dTTP (1 mM) and digested with trypsin for different amounts of time. The products of digestion were separated in a polyacrylamide gel and analyzed by Western blotting using a monoclonal antibody, Ab156, prepared against the entire *rec*A protein. The results of this experiment are shown in Figure

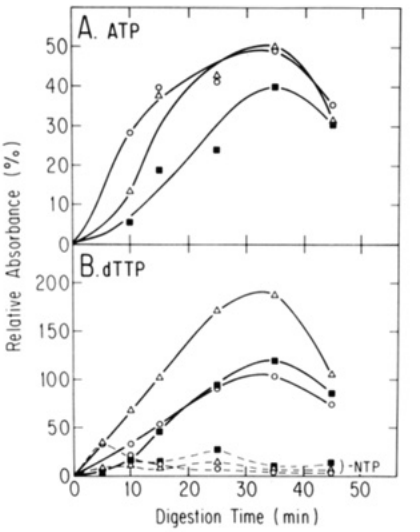

FIGURE 3: Kinetics of fragment accumulation in the presence of ATP or dTTP. *Rec*A protein was incubated with trypsin at an enzyme:substrate ratio of 1:200 (w/w) at 25 °C. Aliquots were withdrawn at the indicated times, and digestion was stopped by addition of gel loading buffer and heating at 100 °C for 2 min. Protein fragments were separated by electrophoresis in SDS–polyacrylamide gels (12% acrylamide, 0.76% cross-link). The relative amounts of fragment 1 (○), fragment 2 (△), and fragment 3 (■) were determined by densitometry of silver-stained gels. Absorbance values were normalized to that of a "constitutive" fragment, the intensity of which was unaltered during the time course of trypsin digestion.

5. The monoclonal antibody reacts strongly with the undigested *rec*A protein as well as two bands which migrate at the positions of bands 1 and 2. A larger, less abundant fragment migrating more slowly than band 1 also reacted with the Ab156 antibody. The identification of these cross-reacting

Table I: Localization of the Antigenic Determinant of *Rec*A Protein Recognized by Ab156 Monoclonal Antibody[a]

*rec*A derivative	plasmid	source/description	cross-reaction with: rabbit anti-*rec*A antiserum	cross-reaction with: monoclonal Ab156
(a) *rec*A+ (residues 1–352)	pBR-*rec*A+	Keener et al. (1984)	+	+
(b) truncated *rec*A (residues 1–259)	pNK100	Sedgwick and Yarranton (1982)/identical with pDR1461	+	–
(c) *rec*A–*lac*Z fusion (residues 1–47)	pGE245	Weisemann et al. (1984)/identical with pGE113	+	–
(d) *rec*A–*lac*Z fusion (residues 1–308)	pGE271	Bremer et al. (1984)	+	–
(e) *rec*A–*lac*Z fusion (residues 260–352)	pJC917	A. J. Clark (personal communication); this laboratory	+	+

[a] Western blot analysis was performed on crude soluble extracts from strains carrying truncated *rec*A genes or in-frame fusions of the *rec*A gene with the *E. coli lac*Z gene. The amino acid residues of *rec*A protein contained in the fusion or truncation product are indicated in parentheses. Proteins were separated by electrophoresis in SDS–polyacrylamide gels (11%, 0.76% cross-linking) and transferred to nitrocellulose as described under Methods. A polyclonal rabbit antiserum prepared against *rec*A protein was used as a positive control. A positive reaction is indicated by (+); no reaction is indicated by (–). The *rec*A–*lac*Z fusion plasmids pGE245 and pGE271 encoded proteins with molecular weights of approximately 118K and 146K, respectively. The molecular weight of the fusion protein encoded by plasmid pJC917 was approximately 10K.

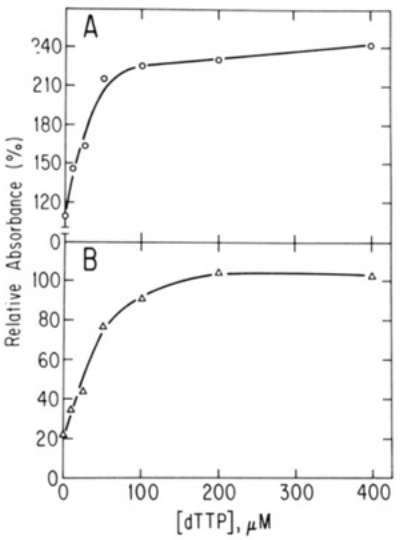

FIGURE 4: dTTP concentration-dependent increase in the accumulation of fragments 2 and 3. *Rec*A protein was incubated with the indicated concentration of dTTP and digested with trypsin for 20 min at 25 °C at an enzyme:substrate ratio of 1:200 (w/w). The absorbance of fragments 2 and 3 was determined by densitometry of silver-stained SDS–polyacrylamide gel lanes and normalized to the absorbance of a "constitutive" fragment as in Figure 3. The relative intensities of fragments 2 and 3 are shown in panels A and B, respectively.

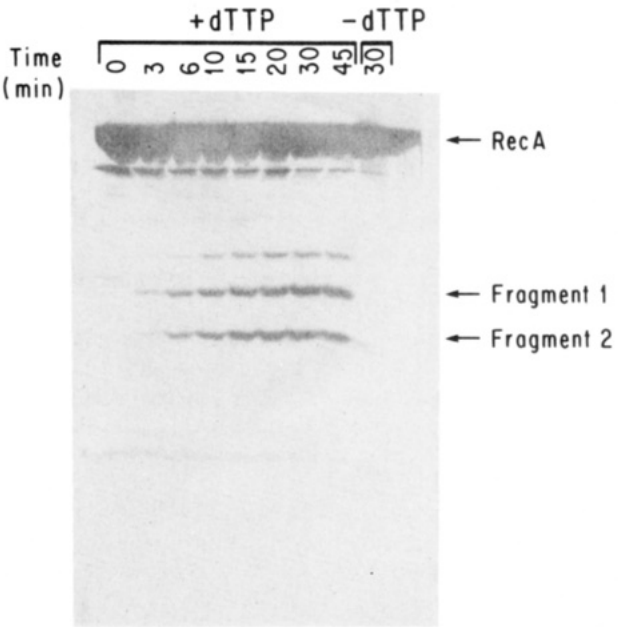

FIGURE 5: Monoclonal antibody Ab156 reacts with tryptic products 1 and 2. *Rec*A protein was incubated with 1 mM dTTP and digested with trypsin at a enzyme:substrate ratio of 1:200 (w/w). At the indicated times, aliquots were removed from the reaction mix, added to gel loading buffer, and heated to 100 °C for 2 min. The digestion products were separated by electrophoresis in a 13% SDS–polyacrylamide gel (0.9% cross-link), and the peptides were transferred to nitrocellulose by electroblotting. The blot was incubated with monoclonal antibody Ab156 and then processed for staining as described (Keener et al., 1984). The –dTTP lane contains a 30-min digestion sample of *rec*A protein without nucleotide.

fragments with bands 1 and 2 is based on the following evidence: (i) these bands have the same mobility as bands 1 and 2 seen in the silver-stained gels; (ii) these bands are present in digests of *rec*A·dTTP but not in digests of *rec*A protein alone; (iii) these bands accumulate with the same kinetics and reach a maximum level after 30–40 min of trypsin digestion. The slower migrating band which we detected by antibody staining is not apparent in the silver-stained gels perhaps due to comigration with other digestion products. These results demonstrate that bands 1 and 2 are overlapping tryptic fragments.

After 30 min of trypsin digestion of *rec*A protein without nucleotide, we detected little or no cross-reacting material at the positions of bands 1 and 2 although the silver-stained gels showed peptides at these positions (compare Figures 1 and 5). These results suggest either that there are unrelated fragments migrating at the same positions as bands 1 and 2 in digests of *rec*A protein or that the sensitivity of detection by the Ab156 antibody is limited.

The region of *rec*A protein recognized by the Ab156 monoclonal antibody was determined in a series of Western blotting experiments using both *rec*A–*lac*Z protein fusions and truncated *rec*A proteins encoded by recombinant plasmids. The results of these experiments are summarized in Table I. The monoclonal antibody did not react with *rec*A–*lac*Z protein fusions containing the first 308 residues of *rec*A protein. The Ab156 antibody also failed to cross-react with a truncated *rec*A protein containing the first 258 residues of *rec*A. All of these fusion and truncation products reacted with rabbit antiserum prepared against *rec*A protein. The fusion protein encoded by plasmid pJC97 contains the first six residues of β-galactosidase fused in frame to the carboxyl-terminal 92 residues of *rec*A protein (residues 260–352) regulated by the *lac* operator and promoter. In extracts of cells containing this plasmid, we detected a protein of the expected size (approximately 11 kDa) using the Ab156 antibody. Taken together, these results argue that the monoclonal antibody Ab156 recognizes one or more determinants distal to residue 307 in *rec*A protein and therefore is a useful probe for the carboxyl-terminal region of *rec*A protein. Recently, Krivi et al. (1985) reported the characterization of an anti-*rec*A monoclonal

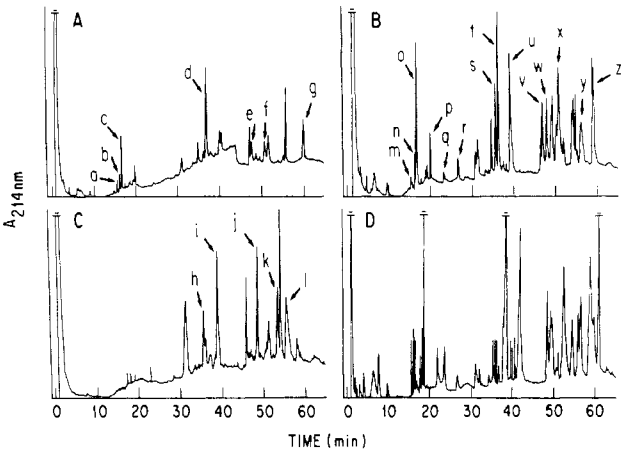

FIGURE 6: HPLC separation of tryptic peptides from bands 1–3 and *rec*A protein. Bands 1–3 were electroeluted from gel slices and digested to completion with trypsin. The digested material was loaded onto a C-18 reverse-phase column and separated as described under Experimental Procedures. Panel A, band 1; panel B, band 2; panel C, band 3; panel D, digested *rec*A protein.

antibody which recognized determinants in the carboxyl-terminal portion of the protein.

HPLC and Amino Acid Composition of Nucleotide-Induced Bands 1–3. In order to more precisely localize and identify the regions of *rec*A protein corresponding to bands 1–3, we isolated the material from preparative SDS–polyacrylamide gels by electroelution (see Experimental Procedures) and digested the peptide material to completion with excess trypsin (1:50 enzyme to substrate, w/w). Digested material obtained for each band was applied separately to a C-18 column, and the peptides were resolved by HPLC. For each band, we compared the HPLC profile of the completely digested material with the profile of completely trypsin-digested *rec*A protein. Under the conditions we have used for HPLC analysis of *rec*A protein, more than 25 of the 42 total tryptic peptides[2] can be resolved, and 23 of these have been identified on the basis of amino acid composition (K. Knight, unpublished results). Thus, by directly comparing the profile of each digested band with that of a complete protein digest, it was possible to further localize the bands in the primary structure. Figure 6 shows the HPLC profiles for bands 1–3 and for fully digested *rec*A protein. As expected, bands 1 and 3 contained a relatively restricted subset of peptides which are also detected in digests of *rec*A protein. However, band 2 contained more peptide peaks than would be expected for a unique fragment of 19 kDa. From digests of band 1, we determined the amino acid compositions of seven distinct peptides from the HPLC profile (peaks a–g) and compared them to the composition of the known tryptic peptides of *rec*A protein. In Table II, we have summarized the composition analysis. Peptide T_{26} (residues 233–243) was the most amino-terminal peptide identified among the products in the band 1 digestion. Several additional peptides spanning the interval between T_{26} and the carboxyl terminus (T_{42}) were detected by amino acid analysis. Thus, on the basis of amino acid analyses, band 1 contains peptides spanning residues 233–352 of *rec*A protein. On the basis of the mobility of band 1 in polyacrylamide gels, we calculated an approximate molecular weight of 22K. From this size estimate, we would expect that the amino terminus of band 1 should be located near residue 150 and, therefore, contain tryptic peptides T_{15} (residues 153–169) to T_{42}. None of the peptides we analyzed was located closer to the amino terminus than peptide T_{26} probably due to the relatively small amounts of band 1 that were recovered for amino acid analysis (less than 0.5 nmol). Therefore, unless band 1 migrates anomalously in polyacrylamide gels containing SDS, we conclude that this partial digestion product is most likely derived from trypsin cleavage at Lys-152. The two other potential sites of trypsin cutting in this region of the *rec*A protein are at Arg-134 or Arg-169. Cleavage at these sites would produce carboxyl-terminal bands with molecular weights of 23.4K and 19.7K, respectively. Nevertheless, the amino acid composition data are consistent with the Western analysis indicating that band 1 contains the carboxyl terminus of *rec*A protein.

[2] Four "tryptic peptides" are single amino acid residues.

Table II: Tryptic Peptides Identified by Amino Acid Composition[a]

peak	tryptic peptide (residues)	peak	tryptic peptide (residues)
	Band 1		
a	T_{28} (246–248)	e	T_{30} (251–256)
b	T_{36} (298–302)	f	T_{37} (303–310)
c	T_{26} (233–243)	g	T_{31} (257–280)
d	T_{42} (325–352)		
	Band 3		
h	T_{15} (153–169)	k	T_{13} (107–134)
i	T_{8} (61–72)	l	T_{14} (135–152)
j	T_{11} (89–105)		
	Band 2		
m	T_{28} (246–248)	t	T_{42} (325–352)
n	T_{36} (298–302)	u	T_{8} (61–72)
o	T_{26} (233–243)	v	T_{30} (251–256)
p	T_{33} (283–286)	w	T_{22} (217–222)
q	T_{25} (228–232)	x	T_{37} (303–310)
r	T_{23} (223–226)	y	T_{14} (135–152)
s	T_{15} (153–169)	z	T_{31} (257–280)

[a] The partial tryptic fragments 1, 2, and 3 were isolated from preparative SDS–polyacrylamide gels and digested to completion with excess trypsin. The digests were separated by using a C-18 reverse-phase column and amino acid compositions determined for individual peaks as described under Experimental Procedures. Typically, the peptide identification was based on the analysis of more than 0.3 nmol of material isolated from the HPLC column. However, peptides T_{11} and T_{13} were identified by using 0.15 nmol of the HPLC-purified peptide. The numbering of amino acid residues is that of Sancar et al. (1980), and the numbering of tryptic peptides is that of Knight et al. (1984). The peak designations are those shown in Figure 6.

Five well-resolved peaks were isolated from the HPLC-separated tryptic digest of band 3 (peaks h–l), and the amino acid composition of each peak was determined. From this analysis, we identified tryptic peptides T_{8} (residues 61–72), T_{11} (residues 89–105), T_{13} (residues 107–134), T_{14} (residues 135–152), and T_{15} (residues 153–169). These results are consistent with the observation that monoclonal antibody Ab156 fails to recognize band 3 and indicate that this partial tryptic product was derived from the amino-terminal end of *rec*A protein. The apparent molecular weight of band 3, based on its mobility in denaturing polyacrylamide gels, is approximately 17.5K. Recent experiments using a monoclonal antibody directed against the first 12 residues of *rec*A protein indicate that the amino terminus is extremely sensitive to trypsin cleavage in the presence or absence of nucleotides (N. Kobayashi, unpublished results). Band 3 does not contain the epitope recognized by this monoclonal antibody, and therefore, we cannot accurately localized either the carboxyl or the amino terminus of this fragment.

The HPLC profile of peptides from band 2 was complex and indicated that this 19K band was composed of more than one partial digestion product since many more peptides were resolved than would be expected for a unique fragment of this size. Most of the peaks derived from digestion of band 2 could

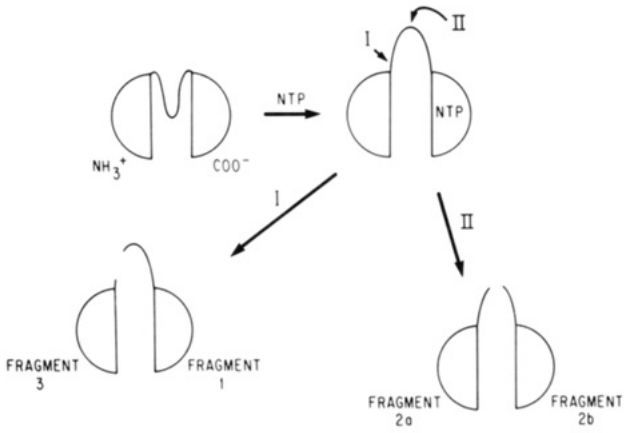

FIGURE 7: Model for generating fragments 1, 2a, 2b, and 3 from *rec*A·NTP complexes. *Rec*A protein binds certain nucleoside triphosphates (NTP's) and undergoes a conformational change. A region of the protein between residues 150 and 180 becomes more accessible to trypsin cleavage in the *rec*A·NTP complex. Cutting at site I by trypsin produces fragments 1 and 3 whereas cutting at site II results in fragments 2a and 2b. More details are provided under Discussion.

be detected in digests of *rec*A protein, suggesting that these extra peptides in band 2 material were not the result of incomplete trypsin digestion of the gel-eluted band. The amino acid compositions of 14 distinct peptide peaks were determined (Table II), and the results indicated that band 2 contained material spanning residues 61 (peptide T_8) to the carboxyl-terminal residue 352 (peptide T_{42}). These peptides were recovered in approximately equimolar amounts following complete digestion of band 2 material, a result which suggested that band 2 was composed of two identically sized products in equimolar amounts. The simplest explanation for these results which is consistent with the size of the induced band 2 (approximately 19K) is that in the presence of dTTP trypsin cleaves at a site near the middle of the polypeptide chain, producing two products of identical mobility. These two fragments, designated 2a and 2b, represent the amino-terminal and carboxyl-terminal halves of *rec*A protein, respectively. Fragment 2b, therefore, is responsible for the binding of antibody Ab156 to band 2 material since this fragment consists of the carboxyl-terminal half of *rec*A protein.

Although we have been unable to determine the exact sites at which trypsin cleaves the *rec*A protein–NTP complexes to produce fragments 1, 2a, 2b, and 3, we have summarized these results in a digestion pathway that is consistent with the kinetic and composition data and minimizes the number of independent trypsin cleavage events that are affected by NTP binding (Figure 7).

Effects of DNA Binding on the Trypsin Sensitivity of RecA Protein. *Rec*A protein binds single-stranded DNA in the absence of nucleoside triphosphates (McEntee & Weinstock, 1981). In order to determine whether this interaction altered the kinetics or products of limited digestion with trypsin, we preformed complexes of *rec*A protein and single-stranded M13 viral DNA for 10 min at 37 °C and subjected the complexes to partial trypsin digestion as described above. Under conditions where dTTP binding caused a marked increase in the levels of products 1, 2a, 2b, and 3, binding of DNA to *rec*A protein produced little or no change in the pattern of partial products compared to digestion of *rec*A protein alone (Figure 8). However, the fraction of *rec*A protein complexed with DNA was not determined in this experiment.

*Rec*A protein binds extremely tightly to single-stranded DNA when the nucleoside triphosphate analogue ATPγS is

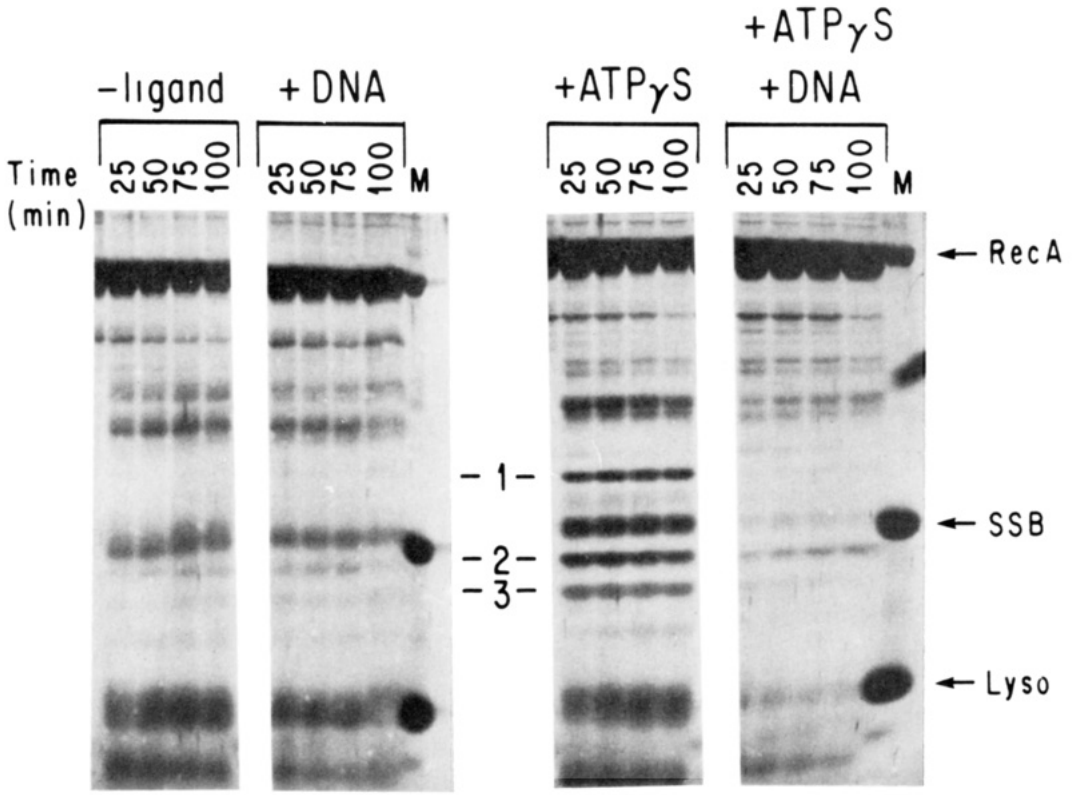

FIGURE 8: Trypsin digestion of *rec*A protein–DNA complexes and *rec*A protein–DNA–ATPγS ternary complexes. *Rec*A protein was preincubated alone (−ligand), with 25 μM M13 viral single-stranded DNA (+DNA), with 200 μM ATPγS (+ATPγS), or with 25 μM M13 DNA and 200 μM ATPγS (+ATPγS + DNA) for 20 min at 37 °C, trypsin was added to a final enzyme:substrate ratio of 1:200 (w/w), and incubations were continued for the indicated amount of time at 25 °C. Aliquots containing 8 μg of *rec*A protein were mixed with gel loading buffer heated at 100 °C for 2 min and electrophoresed in a 13% polyacrylamide gel (0.9% cross-link). Fragments were visualized by silver staining. Markers, *rec*A protein, *E. coli* SSB protein, and lysozyme.

present. Filter binding studies indicate that these stable *rec*A protein–DNA complexes contain tightly bound ATPγS which dissociates extremely slowly from these ternary complexes. In the absence of DNA, however, ATPγS freely dissociates from *rec*A protein (McEntee et al., 1981). In order to determine whether the *rec*A protein in these ternary complexes displayed a trypsin accessibility similar to that of the binary *rec*A protein–ATPγS complexes, we performed *rec*A protein–M13 DNA–ATPγS ternary complexes at 37 °C and then exposed them to low levels of trypsin at 25 °C. The products formed after different times of digestion were separated by electrophoresis in an SDS–polyacrylamide gel and the peptides visualized by silver staining (Figure 8). Digestion of ternary complexes for up to 100 min with trypsin did not produce increased amounts of fragments 1, 2a, 2b, and 3 as observed in the presence of ATPγS alone.

Discussion

The interaction between *rec*A protein and ATP is fundamental to the functions of this enzyme in homologous recombination as well as in the regulation of cellular or viral genes through its proteolytic inactivation of specific repressor molecules, *Lex*A and λcI, respectively. In order for *rec*A protein to drive homologous exchange efficiently, ATP hydrolysis appears to be required whereas only ATP binding is sufficient for its interaction with *Lex*A and λcI repressors (McEntee et al., 1979; Craig & Roberts, 1980; Little et al., 1980).

We have examined the effects of NTP binding to *rec*A protein by investigating the sensitivity and accessibility of the enzyme to trypsin cleavage when it is complexed with different nucleotides. Our results demonstrate that trypsin susceptibility is significantly altered by the binding of different NTP's and analogues which are known to bind the ATP binding site of the enzyme. Specifically, when *rec*A protein is complexed with dTTP, dATP, ATP, or ATPγS, four partial tryptic cleavage products accumulate in digests of the enzyme which are greatly reduced or absent in digests of uncomplexed protein. Little or no change in digestion pattern is detected when *rec*A protein is incubated with AMPPNP, an ATP analogue which binds poorly to the protein. By densitometry of silver-stained gels of *rec*A protein digests, we have demonstrated a saturable dependence of fragment accumulation on the concentration of dTTP. The concentration of dTTP at which we observed a half-maximal enhancement (approximately 50 μM) is consistent with the K_I of dTTP for DNA-dependent ATP hydrolysis by *rec*A protein. These results argue that binding of dTTP (or other NTP's) to the primary ATP binding site of *rec*A protein is responsible for the changes produced in trypsin susceptibility.[3]

The apparent molecular weights of bands 1, 2, and 3 as judged by their mobility in denaturing polyacrylamide gels are 22K, 19K, and 17.5K, respectively. There are two general means by which NTP binding could lead to an accumulation of these bands: (i) NTP binding could *increase* the rate of trypsin cleavage at specific sites, or (ii) NTP binding could *decrease* the rate of degradation of these partial digestion products. An increased rate of trypsin cleavage in specific regions of *rec*A protein could result from a conformational change in the polypeptide accompanying NTP binding. The conformational alteration would render regions of the enzyme more accessible to the protease. Alternatively, a decreased rate of digestion by trypsin might result from conformational effects or from a direct interaction of the NTP's with the partial digestion products that would stabilize them against trypsin processing.

Our digestion results do not unambiguously rule out either of these two possible pathways. The digestion results shown in Figures 1 and 2 do not demonstrate any significant differences in the levels of higher molecular weight products in digests of *rec*A protein compared to *rec*A–NTP complexes. Furthermore, the monoclonal antibody binding experiment shown in Figure 5 fails to reveal any large partial digestion products that are present at higher levels in digests of *rec*A protein alone. These results would argue that bands 1–3 are not produced by increased tryptic action on intermediates that are smaller than *rec*A protein; rather, they are derived from increased proteolytic cleavage at specific sites in intact *rec*A protein–NTP complexes.

The results shown in Figures 1 and 2 are also inconsistent with decreased cleavage of bands 1–3 when *rec*A protein is complexed with NTP's. We do not observe increased levels of smaller tryptic fragments in digests of *rec*A protein compared to digests of *rec*A protein–NTP complexes. Furthermore, staining the gel-separated partial digests with monoclonal antibody Ab156 (Figure 5) or an anti-*rec*A polyclonal antiserum (unpublished results) does not indicate that smaller peptides accumulate in digests of *rec*A protein relative to *rec*A protein–NTP complexes. However, these results apply to peptides with molecular weights of 10K or more since smaller peptides would not be resolved in our polyacrylamide gels and would transfer poorly to nitrocellulose during Western blot analysis.

We have employed monoclonal antibody binding, HPLC separation of tryptic peptides, and amino acid composition analyses of peptides derived from individual gel-isolated bands. Using these data, together with the apparent molecular weights of the bands determined by their relative mobilities in polyacrylamide gels, we have partially localized the regions of *rec*A protein which give rise to bands 1, 2a, 2b, and 3. The size and partial composition of band 1 are consistent with a tryptic intermediate extending from residue 153 to the carboxyl terminus (residue 352). The amino acid compositions of seven peptides derived from complete tryptic digestion of band 1 material indicated that this partial product included the carboxyl-terminal region of *rec*A protein. This result was further supported by the binding of the Ab156 monoclonal antibody to band 1. The HPLC profile and amino acid composition data obtained for peptides from band 2 are most readily explained if this band is composed of two equally sized fragments in equimolar amounts. The apparent size of band 2 (19K) would suggest that a single trypsin cleavage near the center of *rec*A protein was responsible for producing these fragments. Band 3 is derived from the amino-terminal half of *rec*A protein. The size and partial composition of this band suggest that this fragment is derived from trypsin cutting near residue 162. The two closest sites for trypsin cutting are Lys-152 and Arg-169, and on the basis of considerations of the relationship among the four fragments, we suggest that Lys-152 is the more likely site of cutting (see below).

Although we have not been able to precisely locate the important trypsin cleavage sites, our data do demonstrate that the likely residues are at a considerable distance from Tyr-264, the unique site of N_3ATP photoattachment, in the primary structure. It is possible that in the tertiary structure of *rec*A

[3] Similar partial proteolysis experiments were performed by using either *Staph. aureus* V8 protease or α-chymotrypsin. However, we observed no significant differences in the patterns of fragments produced in the absence or presence of nucleotides.

protein, Tyr-264 is in close proximity to regions of *rec*A protein which show altered trypsin susceptibility when NTP's are present. However, we favor a model in which NTP binding to the ATP binding site (containing Tyr-264) induces a change in the conformation of *rec*A protein. Specifically, a region near the middle of the primary structure (residues 150–180) becomes significantly more accessible to trypsin cutting. It is evident from our results that the two regions flanking this exposed "linker" region are considerably more resistant to tryptic cleavage. As demonstrated in Figure 1, these structural domains can be detected after more than 45 min of trypsinization. We propose a "pathway" for the production of fragments 1, 2a, 2b, and 3 from *rec*A·NTP complexes (Figure 7). Cleavage of the polypeptide at Lys-152 would produce two fragments with expected molecular weights of 16.3K (amino-terminal fragment) and 21.5K (carboxyl-terminal fragment). This pattern is consistent with bands 3 and 1, respectively. Alternatively, trypsin cleavage of intact *rec*A protein at or near Arg-176 would produce two fragments each with a molecular weight of 18.9K, in agreement with the mobility and composition of bands 2a and 2b. Although this scheme is consistent with both the size and kinetic data, there are other models that can explain our results.

Two additional observations have been made regarding the specificity of the effect of NTP's on trypsin accessibility. First, we do not detect any differences in digestion pattern when *rec*A protein–M13 single-stranded DNA complexes are exposed to trypsin. Thus, we conclude that the binding of *rec*A protein to DNA is fundamentally different from its binding to NTP's. Second, although binding of ATPγS alters the accessibility of *rec*A protein to trypsin, addition of single-stranded DNA abolishes this effect. Under the conditions of these experiments, the *rec*A protein is part of a stable ternary complex containing ATPγS and DNA. These latter results tend to argue against the simple notion that NTP's complex with specific Lys and Arg residues in *rec*A protein (blocking cleavage at these sites) and stabilize a small number of digestion intermediates. If this was the mechanism by which NTP's altered the mode of trypsin cleavage, we would expect to see accumulation of bands 1–3 in digests of the ternary complexes.

Taken together, our results suggest that the nucleotide binding promotes structural changes in the *rec*A protein. One possibility is that NTP binding causes the *rec*A protein to "open" in a limited region near the center of the protein, giving greater flexibility to the polypeptide. Moreover, our results clearly demonstrate that the amino-terminal and carboxyl-terminal halves of *rec*A can be cleaved apart and retain structural stability. Recently, Blanar et al. (1984) have proposed a structure for the core of *rec*A protein based on computer analysis and comparison to α/β proteins that bind nucleotides. According to their analysis, residues 144–165, which are contained within the segment showing altered trypsin susceptibility, are in a "linker region" connecting a short β segment (β4) with an α-helical region (α4). Interestingly, this segment is also the location of a mutational change ($Gly_{160} \rightarrow Asp_{160}$) corresponding to the *rec*A1 mutation. The defective *rec*A protein encoded by the *rec*A1 allele binds but does not hydrolyze ATP (Rusch et al., 1985) and fails to form complexes with DNA in the presence but not in the absence of ATP (Wabiko et al., 1983). Our model could explain the defect in the *rec*A1 protein by its inability to undergo a nucleotide-induced conformational change that is necessary for the function of the enzyme.

Recently, Wang and Tessman (1986) have determined the amino acid substitutions in a series of *rec*A proteins showing a constitutive protease phenotype. Several of these mutationally altered *rec*A proteins contained changes in the region of *rec*A protein spanning residues 150–180. On the basis of the properties of these mutants, the authors concluded that this region of *rec*A protein is involved in the nucleoside triphosphate binding domain of *rec*A protein. Furthermore, the analysis of a double mutant (*rec*A1201) as well as the separated single mutations constituting this allele suggests that this region of the *rec*A protein (region 2) interacts with the distal region 3 (near residue 300) of the polypeptide. The results presented in this paper are consistent with these genetic observations and provide direct physical evidence for the involvement of region 2 in *rec*A protein–ATP interactions.

Finally, Kuramitsu et al. (1984) demonstrated protection of two cysteine residues in *rec*A protein by ATP or ADP when the protein was exposed to DTNB. These two residues (Cys-90 and Cys-129) are located at a considerable distance in the primary structure from the region of *rec*A protein that is the site for photoaffinity labeling with the ATP analogue 8-N_3ATP, Tyr-264. Our results suggest that the nucleotide-mediated protection of Cys-90 and Cys-129 from DTNB modification is due to a conformational change that renders these residues less accessible to alkylating agents.

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Registry No. dTTP, 365-08-2; dATP, 1927-31-7; ATP, 56-65-5; ATPγS, 35094-46-3.

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DNA Repair Catalyzed by *Escherichia coli* DNA Photolyase Containing Only Reduced Flavin: Elimination of the Enzyme's Second Chromophore by Reduction with Sodium Borohydride†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains FAD plus a partially characterized, second chromophore. In vivo, the flavin is fully reduced ($FADH_2$), but oxidation to a stable, blue radical ($FADH^{\bullet}$) occurs during enzyme isolation. The second chromophore is irreversibly reduced by reaction of the enzyme with sodium borohydride or by photoreduction in the presence of dithiothreitol. A similar reaction occurs with the protein-free chromophore and sodium cyanoborohydride. Reduction of the second chromophore is accompanied by a complete loss of the chromophore's visible absorption and fluorescence but does not significantly affect catalytic activity. The results show that the enzyme can repair dimers by a pathway involving only $FADH_2$. Enzyme-bound $FADH_2$ is fluorescent and exhibits emission (505 nm) and absorption (360 nm) maxima similar to that expected for a 1,5-dihydroflavin derivative. It is proposed that dimer cleavage via the second chromophore independent pathway involves electron donation from excited $FADH_2$ to pyrimidine dimer. Pyrimidine dimer radicals are unstable and spontaneously monomerize. Unmodified second chromophore can also act as a sensitizer in a pathway that requires $FADH_2$. This pathway may be similar to that proposed for the second chromophore independent reaction except that excited $FADH_2$ would be produced via energy transfer from the excited second chromophore. The fluorescence observed for enzyme-bound, unmodified second chromophore is quenched by $FADH^{\bullet}$ and increases 6-fold when the latter is reduced, but the absorption spectrum (λ_{max} = 390 nm, $\epsilon_{390} = 12.7 \times 10^3$ M^{-1} cm^{-1}) is independent of the redox state of the flavin. The latter observation indicates that, if the enzyme-bound chromophores constitute a single molecule, they must be separated by an "insulating" link.

The principal damage resulting from exposure of DNA to ultraviolet light is the formation of cyclobutane dimers between adjacent pyrimidine residues. Photoreactivating enzymes (DNA photolyases) repair UV-damaged DNA by splitting dimers in a rather unusual reaction that requires visible light. DNA photolyase from *Escherichia coli* contains FAD[1] plus a partially characterized fluorescent, second chromophore. In

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; $FADH^{\bullet}$, flavin adenine dinucleotide neutral radical; $FADH_2$, fully (two electron) reduced flavin adenine dinucleotide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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