Direct ATP Photolabeling of *Escherichia coli* RecA Proteins: Identification of Regions Required for ATP Binding

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ABSTRACT: When the *Escherichia coli* RecA protein is UV irradiated in the presence of [α-^{32}P]ATP, a labeled protein–ATP adduct is formed. All the experimental evidence indicates that, in forming such an adduct, the ATP becomes specifically immobilized in the catalytically relevant ATP binding site. The adduct can also be identified after irradiation of *E. coli* cell lysates in a similar manner. This direct ATP photolabeling of RecA proteins has been used to identify regions of the polypeptide chain involved in the binding of ATP. The photolabeling of a RecA protein that lacks wild-type carboxy-terminal amino acids is not detectable. A RecA protein in which the amino-terminal sequence NH_2-Ala-Ile-Asp-Glu-Asn- is replaced by NH_2-Thr-Met-Ile-Thr-Asn-Ser-Ser-Ser- is only about 5% as efficiently photolabeled as the wild-type protein. Both of these RecA protein constructions, however, contain all the elements previously implicated, directly or indirectly, in the binding of ATP. ATP-photolabeled RecA protein has also been chemically cleaved at specific amino acids in order to identify regions of the polypeptide chain to which the nucleotide becomes covalently photolinked. The evidence is consistent with a region comprising amino acids 116–170. Thus, this work and that of others suggest that several disparate regions of the unfolded polypeptide chain may combine to form the ATP binding site upon protein folding or may influence binding through long-range effects.

The *recA*$^+$ gene of *Escherichia coli* is required for homologous genetic recombination and for the induction of the multigene SOS response to DNA damage (reviewed in Clark, 1973; Walker, 1984, 1985). The biochemical properties of the purified RecA protein are generally consistent with its recombinational and regulatory activities in vivo (reviewed in Radding, 1978, 1982; McEntee & Weinstock, 1981; Howard-Flanders et al., 1984). It catalyzes the renaturation of homologous DNA single strands (Weinstock et al., 1979), strand exchange between duplex and homologous single strands of DNA (Shibata et al., 1979; McEntee et al., 1979; Cox & Lehman, 1982), reciprocal strand exchange between gapped circular and linear DNA duplexes (West et al., 1981a,b; DasGupta et al., 1981), and specific proteolytic cleavage of the LexA repressor protein or promotion of its autocatalysis (Little et al., 1980; Little, 1984). All these reactions require DNA-dependent hydrolysis of ATP by the RecA protein or its interaction with the nonhydrolyzable analogue ATP(γS)[1] (Roberts et al., 1978; Ogawa et al., 1978).

This catalog of activities indicates that the RecA protein must interact with several substrates and cofactors. As a result, RecA protein has sometimes been described in terms of separate, structural domains. This view may have originated in studies mapping *recA* gene mutational sites, comparing their different phenotypes, and determining the enzymological properties of their proteins in vitro and in sequence studies (Morand et al., 1977; Sancar et al., 1980; Kawashima et al., 1984). That such a view is an oversimplification is illustrated by the variety of sites that have been proposed for the RecA ATP binding site. A region between amino acids 90 and 130 was proposed on the basis of the ATP-dependent reactivity of Cys^{90} and Cys^{129} (Kuramitsu et al., 1984), while Ser^{70}-Thr^{73} was predicted from an amino acid sequence comparison of several ATP-binding enzymes (Walker et al., 1982). Tyr^{264} has been most convincingly identified as the site of covalent attachment to nucleotide after either photoaffinity labeling with 8-azidoATP or modification by 5′-FSBA (Knight & McEntee, 1985a,b). In agreement with this, it has been shown that a RecA protein lacking some 93 carboxy-terminal amino acids cannot be photoaffinity labeled by 8-azidoATP (Rusche et al., 1985). In this paper, we describe studies on the direct photolabeling of intact and genetically engineered derivatives of the RecA protein by ATP itself. These studies demonstrate the importance of both the carboxy and amino termini of the protein for competent ATP binding. We have also cleaved the RecA protein at specific amino acids in order to determine to which regions of the polypeptide chain the ATP becomes covalently linked.

Materials and Methods

(*a*) *Materials.* [α-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [^{35}S]methionine were from Amersham International. Urea, guanidinium chloride, HBr ("Aristar" grade), dimethyl sulfoxide ("spectroscopic" grade), and "Electran" protein molecular weight markers were from BDH Chemicals Ltd. Nalidixic acid, cyanogen bromide, 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate, and SDS were from Sigma; acrylamide, *N*,*N*′-methylenebis(acrylamide), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from Bio-Rad. ATP, ADP, ATP(γS), and adenosine were from Boehringer Mannheim. Sephadex G-25 (medium grade) was from Pharmacia P-L Biochemicals and 2-nitro-5-(thiocyanato)-benzoic acid from Fluka AG. Nitrocellulose filters (BA85) were from Schleicher & Schüll. The RecA protein was purified essentially as described by Weinstock et al. (1981a).

(*b*) *Bacteria and Plasmids.* These are listed in Table I. Plasmids pPD401, pPD422, and pPD407 were constructed and very generously supplied by Paul Debenham (MRC Harwell).

[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATP(γS), adenosine 5′-*O*-(3-thiotriphosphate); 8-azidoATP, 8-azidoadenosine 5′-triphosphate; 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; TCA, trichloroacetic acid; $NaPP_i$, tetrasodium pyrophosphate; SDS, sodium dodecyl sulfate; M_r, relative mobility; bp, base pair(s).

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Table I: *E. coli* Strains and Plasmids Used

E. coli	relevant genotype	plasmid	source/ref
WP2		pMH1	Sedgwick and Yarranton (1982)
WP2		pMH21	Yarranton and Sedgwick (1982)
WP2		pMH27	Yarranton and Sedgwick (1982)
WP2		pMH29	Yarranton and Sedgwick (1982)
WP2		pDR1461	Sancar and Rupp (1979)
WP2		pBR322	Bolivar et al. (1977)
DM1421	*lexA51 recA*$^+$		D. W. Mount
DM1411	*lexA51 recA121*		D. W. Mount
GY4332	*lexA51* Δ(*srl–recA*)		R. Devoret
KM4104	Δ(*srl–recA*)	pUC12	P. Debenham
KM4104	Δ(*srl–recA*)	pPD401	P. Debenham
KM4104	Δ(*srl–recA*)	pPD407	P. Debenham
KM4104	Δ(*srl–recA*)	pPD422	P. Debenham

They carry *recA* sequences deleted at their 5′-ends and fused to the *lac* transcriptional unit in the vector pUC12. In pPD401 and pPD422 the deletions stop 37 and 13 bp, respectively, before the start codon of the *recA* structural gene. In pPD407 the deletion stops 17 bp after the start. The amino acid and nucleotide sequence numerology is that used by Sancar et al. (1980). Bacterial cultures were grown in Davis and Mingioli (1950) minimal medium under conditions described previously (Sedgwick & Yarranton, 1982). To induce RecA protein synthesis and label cellular proteins, 100-mL cultures of mid-log phase cells were divided into halves. One half was treated with 40 μg/mL nalidixic acid, and after 30-min incubation, 0.5-mL aliquots of both the induced and uninduced control cultures were incubated for 30 min in the presence of [^{35}S]methionine. Unlabeled and labeled cells were collected by centrifugation and the labeled cells lysed for electrophoresis (Sedgwick & Yarranton, 1982). Unlabeled cells were suspended in 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 10% glycerol, and the suspension was passed through a French pressure cell at 4 °C and 18 000 psi. Cellular debris was removed by centrifugation at 4 °C. The supernatant solution was stored at −80 °C and was used for the photolabeling experiments.

(*c*) *Direct ATP Photolabeling. E. coli* cell lysate (50 μg of protein) was irradiated in 100 μL of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 4 μM ATP and containing 15 μCi of [α-^{32}P]ATP. Irradiation was carried out for 20 min in wells of a 96-well microtiter plate held on ice beneath a Hanovia germicidal lamp (low-pressure Hg vapor, 254 nm) delivering a dose of 0.99 mW/cm^2 (determined by a UVX digital radiometer) to the reaction mixture. After irradiation, 5 μL of 50 mM ATP followed by 15 μL of 100% TCA–7.5% $NaPP_i$ was added to each mixture and the precipitated protein held on ice for 30 min. The suspension was microfuged and the pellet washed three times with 0.4 mL of 7% TCA–1% $NaPP_i$ and then dissolved in 65 mM Tris-HCl, pH 8.0, 1% SDS, 1% 2-mercapthoethanol, and 20% glycerol (sample buffer). Proteins were denatured in a boiling water bath for 2 min immediately before electrophoresis. Purified RecA protein at 140 μg/mL was irradiated as above for 20 min in 100 μL of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiotheitol, and 0.1 mM ATP and containing 10 μCi of [α-^{32}P]ATP. The precipitated protein was washed once with 7% TCA–1% $NaPP_i$ before being dissolved in sample buffer. RecA protein at 200 μg/mL destined for chemical cleavage was irradiated for 10 min a reaction mixture containing 30 μCi of [α-^{32}P]ATP. The precipitated protein was washed twice with diethyl ether and dried. Although dithiothreitol can scavenge free radicals, it was not omitted from reactions in order to retain photolabeling conditions close to optimal.

(*d*) *Cleavage of RecA Protein.* Buffer changes of protein solutions during or after completion of cleavage reactions were by centrifugation through gel filtration columns (Helmerhorst & Stokes, 1980). After equilibration of 0.9-mL columns of Sephadex G-25 in 1-mL disposable syringes, the protein solution (100 μL) was centrifuged through. All centrifugations were at 3200 rpm for 4 min in a Sorvall HB4 rotor. Control experiments confirmed that the required buffer and pH changes were achieved and that polypeptides >2.0 kDa were recovered with >80% yield. The cleavage reaction yields quoted below are based upon recovery of ^{32}P activity. For each cleavage reaction, a duplicate RecA protein aliquot was photoaffinity labeled and electrophoresed in the same gel as the cleaved protein. Yields were then estimated from different exposures of the autoradiograms. All cleavages started with 50 μg of RecA protein that had been photolabeled.

(*i*) *Cleavage at Aspartate–Proline Linkages* (*Landon, 1977*). The ATP–protein adduct was dissolved in 100 μL of 70% formic acid and held at 37 °C for 28 h. The resulting solution was centrifuged through Sephadex G-25 equilibrated with sample buffer. The yield was 40 ± 5%.

(*ii*) *Cleavage at Tryptophan Residues* (*Savige & Fontana, 1977*). The ATP–protein adduct was dissolved in 50 μL of a mixture of glacial acetic acid, concentrated HCl, and dimethyl sulfoxide (14:12:1 v/v). After 30 min at room temperature, 20 μL of 48% HBr in dimethyl sulfoxide (4:1) was added and the solution held for a further 30 min. Water (50 μL) was added, the solution extracted with diethyl ether, the aqueous phase lyophilized, and the residue dissolved in sample buffer. The yield was 55 ± 5%.

(*iii*) *Cleavage at Cysteine Residues.* By the method of Stark (1977), ATP–protein adduct was dissolved in 100 μL of 6 M guanidinium chloride and 0.05 mM dithiothreitol in 0.2 M Tris–acetate, pH 8.0. After 30 min at room temperature, 5 μL of 20 mM 2-nitro-5-(thiocyanato)benzoic acid in 0.2 M Tris–acetate, pH 8.0, was added and the solution incubated at 37 °C for 30 min. It was then centrifuged through Sephadex G-25 equilibrated with 0.1 M sodium borate, pH 9.5, 0.1% SDS and the eluant held at 37 °C for 15 h. The solution was then centrifuged through Sephadex G-25 equilibrated with sample buffer. By the method of Wakselman et al. (1976), ATP–protein adduct was dissolved in 100 μL of 7 M urea, 1 mM 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate, 0.1 M sodium acetate, pH 3.5, and processed as in the first cysteine cleavage method. Yields were 55 ± 5% and 65 ± 5%, respectively.

(*iv*) *Cleavage at Methionine Residues* (*Gross, 1967*). ATP–protein adduct was dissolved in 100 μL of 70% formic acid containing 5 mg of cyanogen bromide. After 20 h at room temperature, the solution was centrifuged through Sephadex G-25 equilibrated with sample buffer. The yield was 60 ± 5%. To cleave RecA protein at both Met and Trp residues, it was first cleaved at Trp as described above, and methionine sulfoxides in the resulting fragments were reduced by dithiothreitol and then treated with cyanogen bromide as described above.

(*e*) *Electrophoresis.* Photolabeled cell lysates or purified RecA protein were electrophoresed through 10% or 12.5% separating gels as described by Laemmli (1970). To quantitate the levels of protein-bound ATP, the labeled RecA protein bands were located by autoradiography, excised from the gel, and dissolved in 2 mL of hydrogen peroxide. Radioactivity was determined after addition of Beckman Ready-Solv EP.

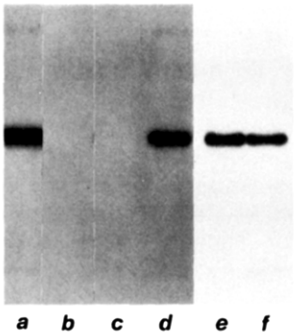

FIGURE 1: ATP photolabeled RecA protein. (a) RecA protein (0.3 nmol) was irradiated with 0.1 mM ATP containing 10 μCi of [α-^{32}P]ATP under standard conditions (see Materials and Methods section); (b) as in (a) but without UV irradiation; (c) as in (a), but the RecA protein was first incubated at 60 °C for 10 min; (d) as in (a), but $MgCl_2$ was omitted from the reaction mixture. In (e) the details are identical with those in (a), but the experiment was done simultaneously with (f), in which 10 μCi of [γ-^{32}P]ATP replaced [α-^{32}P]ATP. The reaction products were electrophoresed through a 10% polyacrylamide gel containing SDS.

Electrophoresis of RecA cleavage products was by the method of Swank and Munkres (1971). The gels were stained in 0.25% Coomassie blue–25% formaldehyde for 60 min followed by 0.25% Coomassie blue–45% methanol–9% acetic acid–0.4% formaldehyde for 3 h (Steck et al., 1980). The stained gels were destained in 50% methanol–10% acetic acid–0.4% formaldehyde before dehydration. Autoradiography was on Fuji RX X-ray film with intensifying screens at −70 °C.

(f) Protein Assays. The DNA-dependent ATPase activity of the RecA protein was assayed as described (Weinstock et al., 1981a). A modified filter binding assay was used in some experiments to quantitate the ATP photolabeling reactions. RecA protein was photolabeled with [α-^{32}P]ATP as described above. The mixture was made 5 mM in ATP and filtered through a nitrocellulose filter, which was then washed as described by McEntee et al. (1979). Control experiments showed that less than 0.005% of the total radioactivity was trapped by the filter in the absence of UV irradiation and that after irradation, the addition of SDS to 0.1% before filtration did not influence the amount of radioactivity trapped. It has been noted that some batches of nitrocellulose filters retain unacceptable control levels of radioactivity. RecA protein concentrations were determined spectroscopically (Weinstock et al., 1981a) and those of other proteins by the method of Bradford (1976).

RESULTS

Direct Photolabeling of Purified RecA Protein. RecA protein became photolabeled when it was UV irradiated in the presence of [α-^{32}P]ATP. The resulting covalent nucleotide–protein adduct could be detected by autoradiography after SDS polyacrylamide gel electrophoresis (Figure 1) or quantitated by filter binding (Figure 2) or by acid precipitation. The electrophoretic mobility of the adduct was identical with that of the unmodified protein. No adduct was detectable when irradiation was omitted or the protein heat inactivated before irradiation (Figure 1a–c). Omission of magnesium ions during irradiation resulted in a 40% decrease in adduct yield (Figure 1d). [γ-^{32}P]ATP photolabeled RecA protein about 85% as efficiently as [α-^{32}P]ATP (Figure 1e,f) while the filter binding assay revealed that [2,8-^{3}H]ATP was equally efficient (not shown). Both ADP and ATP(γS), but not adenosine, inhibited the direct photolabeling by ATP (not shown), while the reaction exhibited saturation kinetics with respect to ATP concentration (Figure 2A).

The extent of photolabeling was proportional to RecA protein concentration over at least a 10-fold range (not shown) and to increasing UV radiation exposure up to about 30 min at the standard fluence, after which there was little further increase in labeling (Figure 2B). This may result from UV inactivation of the RecA protein because its DNA-dependent ATPase activity was reduced by about 45% after 20-min irradiation (Figure 2B). This result also applied to the ATP binding capacity because RecA protein, which had been preirratiated until only 23% of its ATPase activity remained, retained only 26% of its original ability to be photolabeled subsequently by [α-^{32}P]ATP.

The stoichiometry of the photolabeling by ATP was determined by two methods. A value of 0.019 mol of ATP per mol of RecA monomer after 20-min standard irradiation was determined by quantitating the radioactivity in adduct bands excised from a polyacrylamide gel. This is in good agreement with a value of 0.022, determined by the filter binding assay (Figure 2B), and suggests that the complex was relatively stable to acid precipitation.

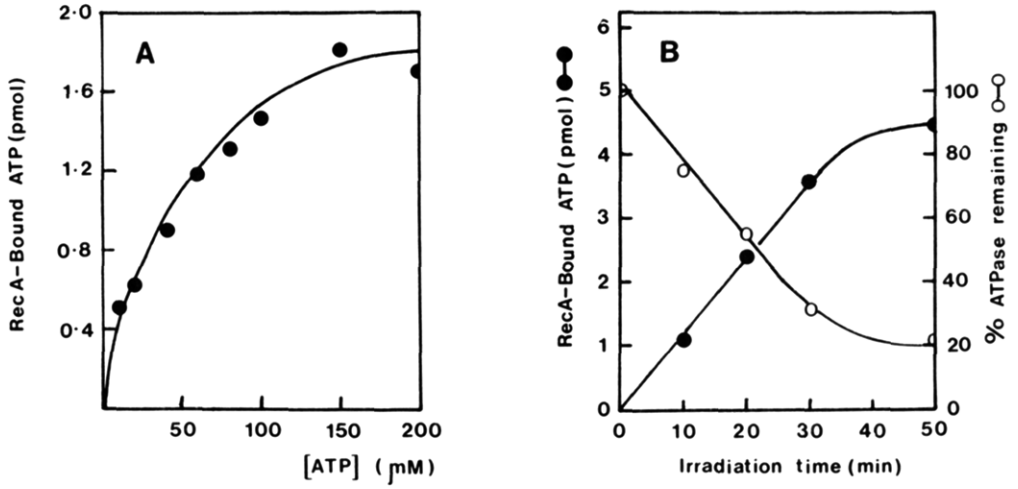

FIGURE 2: ATP photolabeling of RecA protein as a function of ATP concentration and of UV dose. (A) Reaction mixtures contained the ATP concentrations shown, along with the nonbinding adenosine so that total adenosine + ATP concentrations were constant (hence, constant optical density). After irradiation, the [α-^{32}P]ATP covalently bound to RecA protein was determined by the filter binding assay. (B) Reaction mixtures were irradiated for the times shown at the standard dose rate, and the [α-^{32}P]ATP covalently bound to RecA protein was determined by the filter binding assay (●). Reaction mixtures without labeled, but with unlabeled ATP, were irradiated and then assayed for single-stranded DNA-dependent ATPase activity (○). Note that after 20-min irradiation, the protein is 80% inactivated but only 2–3% has been photolabeled.

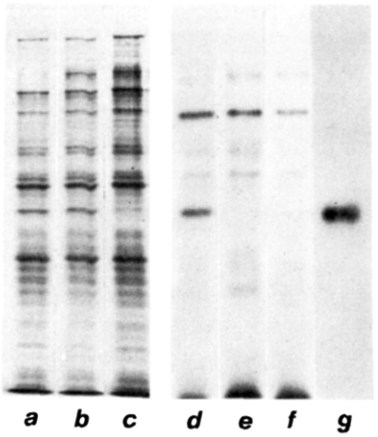

FIGURE 3: ATP photolabeling of wild-type and mutant RecA proteins. Lanes a–c are autoradiograms of in vivo [^{35}S]methionine-labeled proteins in cell lysates and lanes d–f of cell lysates photolabeled with [α-^{32}P]ATP as described in the Materials and Methods section. Lanes a and d are for the *recA* wild-type strain DM1421, lanes b and e for the *recA* base substitution strain DM1411, and lanes c and f for the *recA* deletion strain GY4332. All strains contained the *lexA51* mutation. Lane (g) shows photoaffinity labeled purified RecA protein.

Identification of Photolabeled RecA Protein in Cell Lysates. To identify photolabeled RecA protein in cell lysates, both *recA*$^+$ and *recA* lysates were irradiated in the presence of [α-^{32}P]ATP. In the first experiments, *lexA51* cells with mutationally inactivated LexA repressor were used so that high cellular RecA protein concentrations were made constitutively regardless of their catalytic activity. Photolabeled proteins with a range of M_r values were detectable (Figure 3d). A major one was identified as RecA protein because it coelectrophoresed with authentic photolabeled RecA protein and it was absent from irradiated extracts of *recA* base substitution or deletion mutant strains (Figure 3e–g). After transformation of the deletion strain by a *recA* gene-bearing plasmid, the band reappeared in the photolabeled proteins of the transformant (see Figure 5).

In *lexA*$^+$ cells, the synthesis of RecA protein is induced as a component of the SOS response to DNA damage or replication inhibition. Nalidixic acid is a potent replication inhibitor and efficiently induces the SOS response. A comparison of photolabeled proteins from *lexA*$^+$ cells treated or not treated with nalidixic acid confirmed the above identification of cellular RecA protein by its induction. Thus, the relative intensities and mobilities of the bands were identicial with those of RecA protein identified by [^{35}S]methionine protein labeled in vivo in parallel experiments (see Figure 4 and following section). These experiments, therefore, provide good evidence that photolabeled RecA protein can be identified in polyacrylamide gels after irradiation of cell extracts.

Influence of the Carboxy and Amino Termini on ATP Photolabeling. The ATP binding capacities of truncated RecA proteins lacking 21–40% of their carboxy-terminal amino acids (Sedgwick & Yarranton, 1982; Yarranton & Sedgwick, 1982) were determined by the direct photolabeling of cell lysates. The *E. coli* cells were *lexA*$^+$ and carried a truncated *recA* gene on a plasmid in addition to an intact chromosomal *recA*$^+$. Cell cultures were treated with nalidixic acid or left untreated to serve as controls. During this incubation, small aliquots of both cultures were grown in the presence of [^{35}S]methionine to label proteins in vivo. The resulting cells were lysed for gel electrophoresis. Cells in the major, unlabeled portion of the two cultures were collected and their lysates photolabeled. The induction of the intact, chromosomally encoded RecA protein was observable both directly by [^{35}S]methionine incorporation and by ATP photolabeling in every case (Figure 4). The presence and induction of all four truncated RecA proteins was, however, detectable only by direct in vivo [^{35}S]methionine labeling and not by photolabeling (Figure 4). The constitutive level of RecA protein has been estimated at about 0.5 ng/μg of total cell protein, increasing 10–50-fold after nalidixic acid induction (Karu & Belk, 1982). Thus, 50 μg of total protein used in the photolabeling experiments of Figure 4 should contain 0.5–1.25 μg of induced RecA protein. The photolabeling technique can detect 0.1 μg of purified RecA protein (data not shown), and, for equimolar induced amounts of intact and truncated proteins, truncated proteins with 8–20% of the ATP binding efficiency of that of the intact protein would have been detectable. The [^{35}S] methionine-labeled protein gels of Figure 4 reveal, however, that the molar concentration of the truncated proteins is 2–3-fold greater than that of the intact protein. Therefore, the ATP binding efficiency of the former is expected to be proportionally less than 8–20%, that is, no

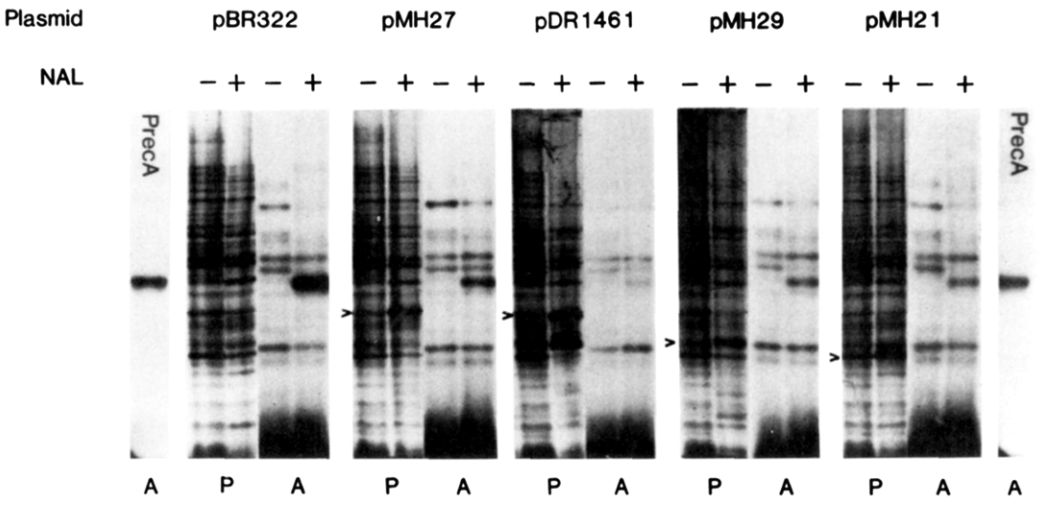

FIGURE 4: ATP photolabeled lysates of *E. coli* cells containing both wild-type and truncated *recA* gene sequences. *E. coli* WP2 contained the following plasmids: pBR322, encodes no RecA protein; pMH27, pDR1451, pMH29 or pMH21, encode RecA proteins lacking 21, 23, 31, and 40% of the wild-type carboxy-terminal amino acids, respectively. Cell cultures were either treated with nalidixic acid (NAL$^+$) or left untreated as controls (NAL$^-$). Lanes marked P are autoradiograms of lysates from the cells labeled in vivo with [^{35}S]methionine; those marked A are autoradiograms of lysates photolabeled with [α-^{32}P]ATP. Both kinds of labeled lysates were electrophoresed on the same 12.5% polyacrylamide gel to ensure accurate matching of bands. The lanes marked PrecA are autoradiograms of the photolabeled purified RecA protein run on the same gel. The open arrows point to the ^{35}S-labeled truncated RecA proteins.

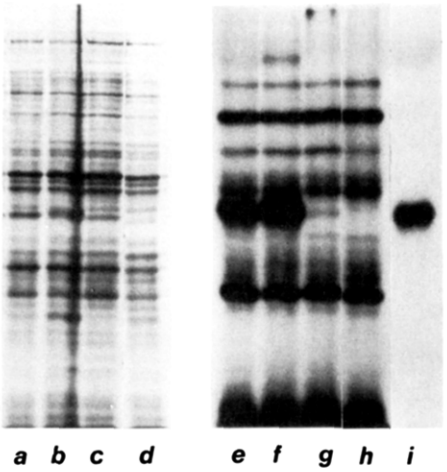

FIGURE 5: Influence of the amino terminus of the RecA protein on its ability to be ATP photolabeled. *E. coli* KM4104 contained the following plasmids: lanes a and e, pPD401-*lacZ* sequence fused to *recA* bp − 38; b and f, pPD422-*lacZ* fused to *recA* bp − 14; c and g, pPD407-*lacZ* fused to *recA* bp + 18; d and h, no plasmid. Lanes a–d are autoradiograms of lysates from cells labeled in vivo with [^{35}S]methionine; lanes e–h are autoradiograms of lysates photolabeled with [α-^{32}P]ATP. Lane i is photolabeled purified RecA protein.

greater than 4–8% of that of the intact protein.

The ATP binding capacity of RecA protein with an altered amino terminus was determined by similar methods. In this case, however, the *E. coli* host cells contained a chromosomal *recA* deletion, so that only multicopy plasmid *recA* sequences were expressed. These sequences had been cloned onto the *lacZ* transcriptional unit at the *Sst*1 site of pUC12. This site was fused to base pairs −38, −14, and +18 (+1 is the A of the initiator ATG) of the *recA* gene sequence in plasmids pPD401, pPD422, and pPD407, respectively (P. Debenham, personal communication). Both pPD401 and pPD422 should express wild-type RecA protein, and possibly longer versions if the *lac* initiator ATG is used. Plasmid pPD407 should encode a *lacZ–recA* fusion product. Figure 5 shows that all three plasmids constitutively expressed an [^{35}S]methionine-labeled protein comparable in size to the authentic *recA* protein, whereas no such protein was present when the host cells carried vector plasmid sequences only (Figure 5a–d). Despite being present in similar molar concentrations (estimated from the [^{35}S]methionine-labeled proten gels), the protein encoded by pPD407 is only about 5% as efficiently photolabeled as the RecA proteins encoded by pPD401 and pPD422 (Figure 5e–h). Thus, the nature of the amino-terminal amino acid sequence can influence the affinity of the RecA protein for ATP.

Chemical Cleavage of Photolabeled RecA Protein. The acid lability of Asp–Pro peptide bonds is well established, cleavage being specific and yielding relatively high M_r polypeptides because of the paucity of Asp-Pro sequences in proteins (Landon, 1977). The RecA protein has one such sequence, Asp^{100}-Pro^{101}, and its cleavage should yield an M_r 10.4K amino-terminal polypeptide and a 27.4K carboxy-terminal polypeptide. An autoradiogram of the acid hydrolysis products of the [α-^{32}P]ATP–protein adduct is shown in Figure 6. The M_r of the major labeled product is in good agreement with 27.4K for the carboxy-terminal polypeptide. A minor band (<3% the intensity of the major one) is present at a position expected for the M_r 10.4K amino-terminal polypeptide. If the relative ^{32}P-labeling intensities of the two equimolar peptide products reflect the ATP binding specificity, the principal binding site should reside on the M_r 27.4K carboxy-terminal polypeptide.

Several procedures have been developed to cleave tryptophanyl peptide bonds. The RecA protein contains two tryptophan residues, Trp^{290} and Trp^{308}, and upon cleavage it should generate an M_r 30.8K amino-terminal polypeptide, a 4.9K carboxy-terminal polypeptide, an internal 1.6K peptide, and partial cleavage products. When the [α-^{32}P]ATP–protein adduct was digested with dimethyl sulfoxide in concentrated HBr (Savige & Fontana, 1977), some 80% of the labeled protein was cleaved (Figure 7). The mobility of the major labeled cleavage product is consistent with it being an amino-terminal polypeptide after cleavage at either Trp^{290} or Trp^{308}. Thus, the principal ATP binding site should be located at least on the larger M_r 32.4K peptide. The Coomassie Blue stained gel from which Figure 7 originated did, in fact, resolve this ^{32}P-labeled region into two closely spaced bands and indicated that the Trp^{290} bond had been cleaved some four times more frequently than the Trp^{308} bond (not shown). Lightly exposed autoradiograms of the gel also indicated that most of the ^{32}P label was associated with the Trp^{290} cleavage product, and so the major ATP binding site can be resolved further to this M_r 30.8K polypeptide.

Proteins can be cleaved at their cysteine residues by cyanylation of their free sulfydryl groups followed by exposure to alkaline pH. In the present work, both 2-nitro-5-(thiocyanato)benzoic acid and 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate were employed as the cyanylating reagents (Stark 1977; Wakselman et al., 1976). The RecA protein contains three Cys residues, Cys^{90}, Cys^{116}, and Cys^{129}. Cleavage should generate an M_r 9.2K amino-terminal poly-

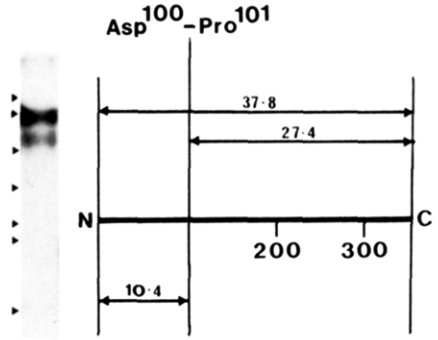

FIGURE 6: Cleavage of [α-^{32}P]ATP photolabeled RecA protein at Asp^{100}–Pro^{101}. An autoradiogram of the electrophoresed reaction products is shown on the left-hand side of the figure and an identification of the labeled polypeptide bands against the RecA protein map on the right-hand side. N and C denote the amino and carboxy termini of the protein, which is marked in units of 100 amino acids. The horizontal arrowed lines are aligned with the corresponding band on the autoradiograms, and their M_r values are as indicated. The arrowheads in this and Figures 7–9 show the mobilities (M_r) of the following marker proteins: ovalbumin, 45K; RecA, 38K; bovine chymotrypsinogen, 25.7K; myogloin, 16.9K; myoglobin I + II, 14.4K; cytochrome *c*, 12.3K; myoglobin I, 8.2K; myoglobin II, 6.2K. In this figure the latter marker was run off the gel.

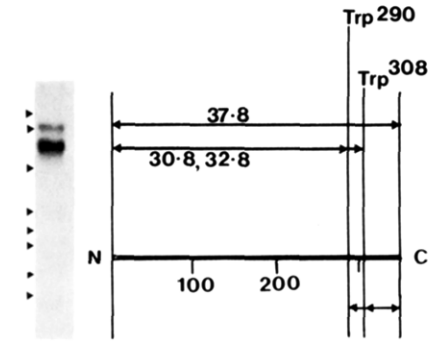

FIGURE 7: Cleavage of [α-^{32}P]ATP photolabeled RecA protein at Trp residues. Figure details are given in the legend to Figure 6.

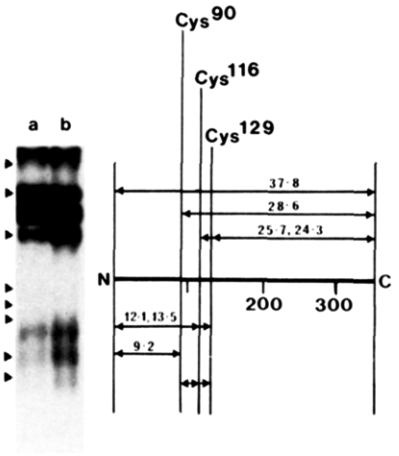

FIGURE 8: Cleavage of [α-^{32}P]ATP photolabeled RecA protein at Cys residues. Figure details are given in the legend to Figure 6. The autoradiogram is somewhat overexposed to show the minor, nonspecific labeling of polypetides. Cyanylation was by (a) 2-nitro-5-(thiocyanato)benzoic acid or (b) 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate.

peptide, a 24.3K carboxy-terminal polypeptide, 2.8 and 1.3K internal peptides, and partial cleavage products. As expected, the two cyanylation methods gave identical labeled products, but cleavage was more efficient with 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate (Figure 8). The M_r values of the major labeled polypeptides agree well with those of the predicted carboxy-terminal M_r 28.6, 25.7, and 24.3K peptides resulting from cleavage at Cys^{90}, Cys^{116}, and Cys^{129}, respectively. The mobilities of the remaining labeled bands identify them as amino-terminal or internal peptides, but their combined labeling intensities are <3% that of the equimolar carboxy-terminal products. These results suggest that the ATP binding site is located on the M_r 24.3K carboxy-terminal peptide.

Cyanogen bromide will selectively cleave proteins at methionine residues under relatively mild acidic conditions (Gross, 1967). The RecA protein contains nine Met residues—Met^{27}, Met^{35}, Met^{58}, Met^{164}, Met^{170}, Met^{171}, Met^{175}, Met^{197}, and Met^{202}. Complete cleavage should, therefore, generate nine peptides as well as homoserine and homoserine lactone arising from the Met^{170}-Met^{171} sequence. In addition, peptides arising from partial cleavage are expected, generating in total a mixture of peptides with a wide M_r range, many of which might be photolabeled. However, the pattern of the labeled cyanogen bromide cleavage products of the [α-^{32}P]ATP–protein adduct is surprisingly simple (Figure 9a). There are two major labeled polypeptides of M_r 11.9 ● 0.5K and 18.0 ± 0.5K, determined from autoradiograms and the corresponding bands on the original stained gels from several experiments. As the smallest possible cyanogen bromide carboxy-terminal polypeptide has an M_r of 16.4K (cleavage at Met^{202}, Figure 9), the 11.9K labeled polypeptide must be amino terminal to Met^{202}. In that region, polypeptides generated by cleavage from Met^{58} to Met^{164}, Met^{170}, Met^{171}, and Met^{175} have M_r values of 11.1, 11.6, 11.7, and 12.2K, respectively, all in good agrement with the 11.91 ± 0.5K determined for the labeled polypeptide. Other possible polypeptides have significantly lower or higher M_r values. An M_r 18.0 ± 0.5K polypeptide, amino or carboxy terminal, can, however, be generated by cleavages at combinations of several Met residues—the intensity of the staining at this mobility may suggest that many such cleavages were indeed realized (Figure 9b). However, evidence consistent with the M_r 18.0 ± 0.5K label being amino terminal was obtained by cleavages at Trp

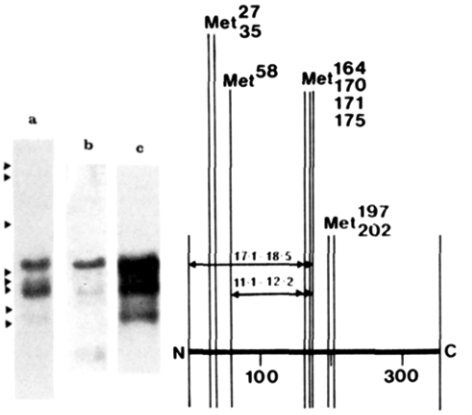

FIGURE 9: Cleavage of [α-^{32}P]ATP photolabeled RecA protein at Met residues. Lane a shows the autoradiogram and lane b the Coomassie Blue stained gel. Lane c shows an autoradiogram of the results of cleavage at Trp and then at Met (see Materials and Methods). Other details are given in the legend to Figure 6.

and then at Met residues. A carboxy-terminal Met polypeptide should suffer a reduction of 4.9 or 6.5K in M_r value by additional cleavage at Trp^{308} or Trp^{290}, respectively. No such reduction was detected (Figure 9c).

Discussion

We have investigated the nature of the ATP binding site of the multifunctional RecA protein of *E. coli* by direct ATP photolabeling methods. Photolabeling of proteins by their nucleotide substrates or by analogues has proved invaluable in a variety of structural studies. These have included identification of substrate binding subunits in multifunctional enzymes, of amino acid specificity of binding, and of specific proteins in cell extracts (Wagenvoord et al., 1977; Yue & Schimmel, 1977; Eriksson et al., 1982; Hollemans et al., 1983; Abraham & Modak, 1984; Biswas & Kornberg, 1984, Woody et al., 1984). Detailed photoaffinity labeling studies of the RecA protein by 8-azidoATP have been made (Knight & McEntee, 1985a–c; Rusche et al., 1985).

It has been established that RecA protein forms a stable 1:1 molar complex with the nonhydrolyzable cofactor ATP-(γS), but only in the presence of single-stranded DNA (Weinstock et al., 1981). The filter binding method used was unable to detect a complex with ATP, whether in the absence or presence of DNA. By nonequilibrium dialysis, however, it was shown that ATP binds reasonably tightly to free RecA protein in the absence of DNA with a 1:1 molar ratio and a dissociation constant of about 30 μM. In addition, over a period of 20 min under reaction conditions similar to those used in the present work, there was negligible ATP hydrolysis (Cotterill et al., 1982). These results provide a reason for our finding, based on the comparable photolabeling efficiencies of [α-^{32}P]-, [γ-^{32}P]-, and [2,8-^{3}H]ATPs that it was ATP and not a degradation product that was covalently linked to RecA protein after UV irradiation. They also support our evidence that the linkage of ATP to the protein reflects a specific catalytically relevant binding reaction rather than a result of random ATP–protein collisions. Thus, although the efficiency of labeling was only 2–3%, presumably a result of the inefficiency of the photolinking reaction, catalytically active protein was an absolute requirement. Heat or UV-inactivated RecA protein and the mutated products of defective *recA* genes were not photolabeled. The pattern of inhibition of the ATP photolabeling by ADP, ATP(γS), and adenosine also reflected their comparative binding affinities determined in other ways (Weinstock et al., 1981a,b). Finally, the saturation kinetics with respect to ATP concentration for photolabeling are

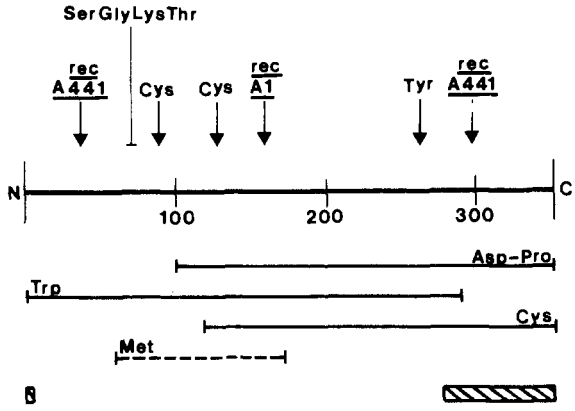

FIGURE 10: Summary of proposed amino acid sequences involved in the interaction of RecA protein with ATP. The sites marked above the line denoting the 352 amino acid RecA protein are those suggested by other workers referred to in the text. The horizontal lines below are the major labeled polypeptides identified after specific cleavages of ATP photoaffinity labeled RecA protein. The hatched boxes are the two terminal sequences required for RecA protein photoaffinity labeling, which have also been identified in this work.

compatible with the observations of others (Weinstock et al., 1981b; Cotterill et al., 1982).

ATP photolabeled RecA protein was also readily detectable among the other labeled proteins after UV irradiation of *E. coli* lysates. Its identity was established by the following criteria: (1) An M_r identical with the in vivo [^{35}S]-methionine-labeled protein and with unmodified or photolabeled purified RecA protein; (2) its absence from lysates of *E. coli* strains carrying a chromosomal *recA* gene deletion and its reappearance when such a strain was subsequently transformed by a *recA* gene carrying plasmid; (3) its inducibility by nalidixic acid. All the carboxy-terminal truncated RecA proteins failed to exhibit detectable direct ATP photolabeling, even though the chromosomally encoded intact protein present was efficiently labeled. The physiological consequences of these truncated RecA proteins on an otherwise *recA*$^+$ cell have been well documented (Sedgwick & Yarranton, 1982; Yarranton & Sedgwick, 1982). They inhibited the activity of the chromosomal RecA protein in in vivo recombination processes (negative complementation) without causing a concimitant inhibition of its regulatory activities. The RecA1 protein is also defective in ATP metabolism in vitro (Ogawa et al., 1978; Rusche et al., 1985) and displays similar negative complementation behavior in vivo when it is present in a cell with wild-type protein (Yancey & Porter, 1984). Although these two sets of observations may well indicate a possible correlation between negative complementation and a defect in ATP hydrolysis, the fact that the DNA-dependent ATPase activity of the RecA protein is required for the majority of its other catalytic activities may obscure the primary cause of the negative complementation.

The plasmid pPD407-encoded RecA protein, in which the amino-terminal sequence NH_2-Ala-Ile-Asp-Glu-Asn- is replaced by NH_2-Thr-Met-Ile-Thr-Asn-Ser-Ser-Ser-, was only about 5% as efficient as the wild-type RecA protein in binding ATP. This result, therefore, defines another region of the RecA polypeptide chain that is required for the successful binding of the ATP substrate.

We have also attempted to identify the ATP binding region with greater resolution. The ATP photolabeled RecA protein was cleaved at specific amino acids, and the products were separated by polyacrylamide gel electrophoresis. The major ATP-labeled polypeptides were then identified by their M_r values. Although the fragments produced by cleavage at Asp–Pro, Trp, or Cys may be reliably identified on the basis of their electrophoretic mobilities, a definitive identification of the Met cleavage fragments has to await peptide mapping and/or sequence data. This is because the amino acids involved in the first three reactions are infrequent enough in the RecA protein to result in a limited number of disparate sized fragments. There are, however, nine Met residues with the result that, when partial cleavage products are also included, many polypeptides of similar size can be generated after cyanogen bromide cleavage. Moreover, Swank & Munkres (1971) have emphasized the importance of not only the molecular size but also charge and conformation in the determination of the electrophoretic mobilities of polypeptides, especially at the lower end of the size range. Nevertheless, the result of the Trp–Met cleavage experiment provides preliminary evidence that the major labeled Met polypeptides are not carboxy-terminal ones.

The Asp–Pro, Trp, or Cys cleavages define a region from amino acids 116 to 290 as including the site(s) of ATP photolabeling (Figure 10). This region also includes the *recA1* mutant site (Kawashima et al., 1984), one of two cysteine residues protected by ATP from modification (Kuramitsu et al., 1984) and Tyr^{264}. Inclusion of the preliminary Met cleavage data further limits the region between amino acids 116 and 170, which includes the *recA1* site and protected cysteine only. However, the elegant work of Knight and McEntee (1985a,b) clearly establishes the importance of Tyr^{264} in the ATP binding site because of induced linkage to 8-azidoATP and to 5′-FSBA, in which the reactive groups are undoubtably oriented differently. Our data suggest direct photolabeling of a different region of the polypeptide by ATP, but where different regions of a polypeptide chain contribute to a binding site, the actual amino acid(s) photolabeled will depend on the nature of the label itself, the chemistry of the photolabeling reaction(s), and the availability of amino acids that can be photolabeled by a particular probe. For example, we cannot rule out a possibility that photoexcitation of the protein itself is required for photolabeling, resulting in protein inactivation and, less frequently, reaction with ATP in the binding site.

The importance of disparate regions of the RecA polypeptide chain in determining ATP binding, is, however, most convincingly shown by the results of the photolabeling experiments on the amino-terminal modified and carboxy-terminal truncated RecA proteins described above. The *recA* construct in plasmid pMH27 has lost all *recA* sequences beyond bp 884 ± 10 (Yarranton & Sedgwick, 1982) and therefore retains all the elements, including Tyr^{264}, which have been implicated directly or indirectly in ATP binding. Out of more than 60 $\gamma\delta$ transpositional insertions into the *recA* gene, none were found beyond bp 884 ± 10 (S. G. Sedgwick, unpublished data). Because such insertions were selected by virtue of their inactivation of the protein (Sedgwick & Yarranton, 1982; Yarranton & Sedgwick, 1982), it is possible that insertions beyond that position do not inactivate or, indeed, prevent ATP binding. Although the two terminal regions may form a part of the ATP binding site, it is more likely that they influence ATP binding through long-range conformational effects within each subunit or possibly disruption of the quaternary protein structure.

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Registry No. ATP, 56-65-5.

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