Photoaffinity Labeling of the recBCD Enzyme of Escherichia coli with 8-Azidoadenosine 5′-Triphosphate*

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The recB and recD subunits of the recBCD enzyme (exonuclease V) from Escherichia coli were covalently photolabeled with the ATP photoaffinity analogue [α-32P]8-azido-ATP. The labeling was specific for ATP binding sites by the following criteria. (i) Saturation occurs at high 8-azido-ATP concentrations with dissociation constants of 30 and 120 μM for the recD and recB subunits, respectively; (ii) ATP strongly inhibits the photolabeling; (iii) 8-azido-ATP is hydrolyzed by the recBCD enzyme and supports its double-stranded DNA exonuclease activity; and (iv) the label is largely confined to two peptides obtained by tryptic digestion of the photolabeled holoenzyme; one is derived from the recB subunit and the other from the recD subunit.

Exonuclease V plays an important role in homologous recombination and in the repair of DNA in Escherichia coli (Muskavitch and Linn, 1981). The enzyme consists of three subunits, the products of the recB, recC, and, as shown recently, the recD genes (Amundsen et al., 1986). It is, therefore, referred to as the recBCD enzyme. In vitro the enzyme has single- and double-stranded DNA-dependent ATPase, ATP-dependent single- and double-stranded exonuclease, ATP-stimulated single-stranded exonuclease, and DNA helicase activities. Of particular interest in attempting to understand the catalytic mechanism of recBCD enzyme is the role of ATP hydrolysis in these various activities. As a first step in examining this question, we have used the ATP analogue 8-N3-ATP in photoaffinity-labeling studies to define the number and subunit location of the sites that bind and hydrolyze ATP.

The photoaffinity technique (Potter and Haley, 1983; Bayley and Knowles, 1977) relies on a substrate analogue that can bind to specific ligand binding sites on a protein, which upon irradiation at the appropriate wavelength produces a reactive moiety which then reacts covalently with the protein. Long wavelength ultraviolet irradiation of 8-N3-ATP produces a reactive nitrene which makes it a useful photoaffinity reagent in the analysis of enzymes utilizing ATP. In fact, it has been used as a probe of, among others, recA protein (Knight and McEntee, 1985; Kowalczykowski, 1986), RNA polymerase (Woody et al., 1984), and DNA polymerase I (Abraham and Modak, 1984).

We report here photoaffinity-labeling studies with 8-N3-ATP and the recBCD enzyme. Both the recB and recD subunits can be labeled by this analogue. The results of titration and nucleotide inhibition experiments, the ability of 8-N3-ATP to serve as a substrate for the recBCD enzyme, and peptide mapping of the covalent modification sites all indicate that both sites are of high affinity and specificity and, therefore, likely to be of importance in the catalytic mechanism of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled 8-N3-ATP was obtained from Sigma. A stock solution was made up to 30 mM in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and stored at −80°C. The 8-N3-ATP concentration was determined by its absorbance at 280 nm using an extinction coefficient of 13.1 mM−1 cm−1 (Haley and Hoffman, 1974). (α-32P)8-N3-ATP was purchased from ICN and ATPγS from Boehringer-Mannheim. Sephacryl-S300 and ATP-agarose (ATP attached through the N6-amino group) were from Sigma. Heparin-agarose, prepared according to Davison et al. (1979) was a gift from J. Kelly of this department.

pMOB45 plasmid DNA was a gift from Hisao Masai, DNAX Research Institute. Tritium-labeled supercoiled M13mp8 DNA (30,000–90,000 cpm/nmol) was prepared as described for M13Gori1 (Julin et al., 1986). Tritium-labeled E. coli DNA was prepared from strain K37. Cultures grown to saturation were lysed with lysozyme and Triton X-100 as in Davis et al. (1980), except that the lysis mixture was vortexed vigorously for 1 min to release the chromosomal DNA from the cell debris after the Triton X-100 treatment. The DNA was purified from the lysate by two CeCl3/ethidium bromide equilibrium centrifugation steps as described in Davis et al. (1980). This procedure yielded 800 μg of DNA/liter of culture with a specific activity of 45,000 cpm/nmol (nucleotide). The product was sonicated for 30 s to reduce it to small fragments (average size ≤ 1000 nucleotides).

Aldolase (rabbit muscle, type IV) and ovalbumin were purchased from Sigma. The aldolase suspension in 2.5 M ammonium sulfate was dialyzed overnight versus 1000 volumes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and the protein concentration was determined by measuring the absorbance at 280 nm, using E1%0 = 9.38 (Rose and O'Connell, 1977). Trypsin (type TRTPCK, 219 units/mg, tosylphenylethylamidase) was from Worthington. BamHI restriction endonuclease was from New England Biolabs.

Purification of recBCD Enzyme—Plasmid pFS11-04, containing the recB, -C, and -D genes, was a gift from Y. Takagi, Kyushu University, Japan (Sasaki et al., 1982). The 19.5-kilobase BamHI fragment of pFS11-04 containing the recBCD genes was inserted into the BamHI site of the runaway replication vector pMOB45 (Bittner and Vapnek, 1981), and the resulting recombinant plasmids (pDAJ1) were used to transform E. coli strain HB101 (Maniatis et al., 1982). Clones containing the desired plasmid were isolated by selecting for the ability to grow on plates containing chloramphenicol (5 μg/ml) but not tetracycline (5 μg/ml). The structure of these plasmids were determined by restriction endonuclease analysis to verify the presence of the 19.5-kilobase BamHI fragment. The ATP-dependent double-stranded exonuclease activity of the recBCD enzyme was overpro-

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1 The abbreviations used are: 8-N3-ATP, 8-azidoadenosine 5′-triphosphate; ATPγS, adenosine 5′-O-(thiotriphosphate); DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); NaDodSO4, sodium dodecyl sulfate.
ducing by as much as 80-fold upon temperature-shift induction of plasmid replication.

The ATP-dependent double-stranded exonuclease activity of the recBCD enzyme was assayed during the purification by the trichloroacetic acid precipitation assay described by Eichler and Lehman (1986) using 30S labeled E. coli DNA (16 μM nucleotide) and 60 μM ATP. Protein concentrations were determined by the method of Bradford (1976).

E. coli strain HB101[pDA1] was grown in 200 liters of LB broth (Maniatis et al., 1982) containing 0.5% cerelose and 5 μg/ml chloramphenicol at 30°C to an absorbance at 595 nm of 0.42, at which time the temperature was shifted to 42°C to induce plasmid replication. Growth was continued for an additional 4 h, and the cells were harvested and frozen in liquid nitrogen.

Cell lysis and ammonium sulfate precipitation were carried out as described by Dykstra et al. (1984), except that the phenylmethylsulfonyl fluoride and sodium bisulfite were not added. The redissolved ammonium sulfate pellet was chromatographed on DEAE-cellulose (Whatman) as in Eichler and Lehman (1977), followed by chromatography on Sephacryl-S300 and heparin-agarose as described by Gracy (1977). Growth was continued for an additional 4 h, and the cells were recovered in a total volume of about 100 ml. Two aliquots of trypsin (16 μg each) were added at 3-h intervals, and the mixture was incubated at 37°C for a total of 6-8 h. NaDodSO4 (1% w/v) and DTT (20 mM) were added, and the mixture was refrigerated until HPLC analysis (see below).

When the photolabeled recB and recD subunits were separated before tryptic digestion (Fig. 14), one-half of the mixture was treated as described above for the holoenzyme, while the remaining half was subjected to NaDodSO4-polyacrylamide gel electrophoresis. The recB and recD bands were cut from the denatured gel, shaken for 5 min at 4°C in 1% w/v SDS, followed by 10% ammonium bicarbonate, pH 8.0 (4 × 1 ml each), and the slices were minced by forcing them through a fine steel mesh. Ammonium bicarbonate (pH 8.0) (0.8 ml of 1%) and trypsin (8 μl of 5 mg/ml) were added and the samples incubated at 37°C. Two more additions of trypsin were made at 3-h intervals, and NaDodSO4 was added to 0.1% (w/v) after a total digestion time of 11 h. The peptides were separated from the gel fragments by centrifugation through Centricon 30 filters. The fragments were washed twice on the filter with 0.6 ml of water. The combined filtrates were concentrated almost to dryness by Speed-vac concentrator and taken up again in 10 μl of water and 10 mM DTT.

HPLC Analysis of Tryptic Digests—Reversed phase chromatography was done using a Vydac C-18 column. Elution buffers were: A, 0.1% (v/v) trifluoroacetic acid; and B, 0.1% (v/v) trifluoroacetic acid and 70% (v/v) acetonitrile. Trypsin-digested [α-32P]N3-ATP-labeled peptides were injected onto the column (equilibrated in 100% A) and eluted by (i) 5 min of 100% buffer A, (ii) a 90-min linear gradient of 0-60% buffer B, and (iii) 10 min of 40% A, 60% B, at a total flow rate of 1 ml/min. Peptides were detected in the eluate by continuous monitoring of the absorbance at 224 nm. Fractions were collected and the radioactivity was determined by Cerenkov counting of the entire fraction.

Two-dimensional Thin Layer Chromatography—Electrophoresis of Peptides Purified by HPLC—Two-dimensional thin layer chromatography was carried out essentially as described by Gracy (1977). Peak fractions were concentrated by freeze drying from methanol (6 inches) at -80°C, evaporated to dryness by Speed-vac concentrator and redissolved in 1 μl of TLC buffer 1 (acetic acid/formic acid/water, 25:25:50, v/v/v) containing 2% 125I methyl green dye. The samples were applied near one corner of a polygram CEL400 (Brinkmann Instruments) cellulose TLC plate (20 × 17.5 cm). Electrophoresis was performed in the first dimension in buffer 1 at 800 volts with the sample spot placed nearest the anode. The plates were then dried thoroughly and chromatographed in the second dimension in butanol/pyridine/acetiacid/water, 32:5:2.5:20, v/v/v/v. Radioactive peptides were visualized by autoradiography.

Nucleotide Hydrolysis Assays—ATPase and 8-NTA-ATPase assays were performed using the thin layer chromatographic method described in Kornberg et al. (1978). Reaction mixtures (20 μl) contained 25 mM PIPES (pH 7.0), 10 mM MgCl2, 110 μM [α-32P]ATP (800 μCi/μM), 110 μM ATP, 0.088 μM [α-32P]N3-ATP (170 μCi/μM), linear duplex pMOB45 DNA (45 μM nucleotides), and recBCD enzyme (0.44 μM). Aliquots (0.5 μl) were removed and applied to polyethyleneimine TLC plates (PEI-CELLULOSE F, MCB Reagents) which were marked off in 0.6 × 5-cm lanes and prespotted with ATP and ADP (0.088 μM, 1 μm for each). After electrophoresis, ATP and ADP markers were located by illumination with shortwave UV light, and the spots were cut out and counted in a toluene-based scintillation fluid. The [α-32P]ATP used in these experiments contained 5.6% ADP initially, while 36% of the radioactivity in the [α-32P]N3-ATP conjugate with the ADP marker, indicating substa-
Photoaffinity Labeling of recBCD Enzyme

Separation of recD Subunit from recBC Complex—The recD subunit was separated from the recBC complex as described by Amundsen et al. (1986). RecBCD enzyme (0.044 mg) and ovalbumin (0.1 mg) were treated with 4 M NaCl at 0 °C for 2.5 h and sedimented by centrifugation for 39 h at 49,000 rpm in a SW 50.1 rotor, 4 °C, through a 10–30% glycerol gradient containing 3 M NaCl and 0.1 mg/ml ovalbumin. Reconstitution of the double-stranded exonuclease activity was also performed as described by Amundsen et al. (1986).

RESULTS

Photoaffinity Labeling of recB and recD Subunits with 8-N3-ATP—The recBCD enzyme was photolabeled by UV irradiation at 0 °C in the presence of [α-32P]8-N3-ATP. As shown in Fig. 1, the recB and recD subunits were strongly labeled after irradiation times of up to 30 s, whereas much less label was incorporated into the recC subunit (lanes 2–5). Aldolase which was used to control for nonspecific labeling, since it does not interact with ATP or other nucleotides, showed only low levels of incorporation. No labeling occurred without UV irradiation (lane 1) even after incubations as long as 5 min (not shown). As judged by densitometer scanning of the autoradiogram, labeling of the recB and recD subunits was 7- and 13-fold greater, respectively, than that of aldolase, on a weight basis, after 30 s of irradiation (more than 20 times more label appeared in recB and recD than in aldolase on a molar basis, calculated using molecular weights of 40,000 (aldolase), 134,000 (recB, Finch et al., 1986a), and 67,000 (recD, Finch et al., 1986b) (Fig. 2). The extent of labeling was as great as 50%, depending on irradiation time, as determined by liquid scintillation counting of bands cut out from the dried gel (data not shown). Both the recB and recD subunits were also photolabeled at 25 °C, although the extent of incorporation was slightly lower than when the experiment was performed at 0 °C (data not shown). Labeling of the recD subunit was virtually abolished by the addition of NaDodSO4 (0.1%) or by preheating the mixture for 2 min at 100 °C prior to irradiation (data not shown). These treatments reduced the labeling of the recB subunit only slightly and led to increased labeling of both subunits.

IRRADIAATION TIME (sec)

0 5 10 20 30

Fig. 1. Photolabeling of recBCD enzyme and aldolase by 8-N3-ATP. Photolabeling was done under standard conditions (see "Experimental Procedures") with 200 μM [α-32P]8-N3-ATP (188 μCi/μmol), recBCD enzyme (0.022 mg/ml), and recD (0.022 mg/ml) in a total volume of 50 μl. Aliquots were removed after 0 (lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), and 30 (lane 5) s of UV irradiation and treated as described under "Experimental Procedures." NaDodSO4-polyacrylamide gel electrophoresis and autoradiography were carried out as described under "Experimental Procedures."

Fig. 2. Relative extents of photolabeling of recB and recD subunits and aldolase. Lanes 2–5 in the autoradiogram in Fig. 1 were scanned by densitometry and the peaks cut out and weighed. The peak weights are plotted per amount of recB or recD subunit and aldolase present in the photolabeling mixture.

Fig. 3. Photolabeling in the presence of DNA and cytidine. Photolabeling was performed under standard conditions with 40 μM (lanes 1–6) or 200 μM (lanes 7–12) [α-32P]8-N3-ATP (218 μCi/μmol) and recBCD enzyme (0.022 mg/ml) in a total volume of 20 μl. Sonicated calf thymus DNA was added at 0.15 mg/ml (lanes 2 and 8), 0.36 mg/ml (lanes 3 and 9), and 0.73 mg/ml (lanes 5 and 11). Cytidine was added at 1.24 mM (lanes 4 and 10) or 2.49 mM (lanes 6 and 12). Irradiation was for 30 s on ice, as described under "Experimental Procedures."
stranded DNA in the presence of ATP (A) or 8-N3-ATP (B). Reaction mixtures contained 25 mM PIPES (pH 7.0), 10 mM MgCl₂, 0.67 mM DTT, 3H-labeled M13mp18 closed circular double-stranded DNA linearized by cleavage with BamHI (20 μM nucleotide, 31,000 cpm/nmol) and recBCD enzyme (0.080 pg/ml) in the presence of 40 nM nucleotides). 3H-labeled M13mp18 closed circular double-stranded DNA was measured as described under "Experimental Procedures." Labeling of the recBC subunit and aldolase, probably as a result of enhanced nonspecific binding of 8-N3-ATP to the denatured proteins. ATP strongly inhibited the photolabeling (see below and Figs. 8–10).

Effect of DNA on Photolabeling—The extent of photolabeling was reduced substantially by high concentrations of sonicated calf thymus DNA (Fig. 3). Cytidine at a concentration giving an absorbance at 280 nm identical to that of the DNA also inhibited photolabeling (Fig. 3) indicating that the inhibition by DNA is at least partially due to the reduced UV flux caused by the high optical density (A₂₈₀ = 16 cm⁻¹) of the solution. Cytidine at these concentrations did not affect the DNA-dependent 8-N3-ATPase activity of the recBCD enzyme (not shown).

Enzymatic Activities of recBCD Enzyme with 8-N3-ATP as Substrate—The ability of 8-N3-ATP to serve as a substrate for the ATPase and as a co-substrate for the double-stranded exonuclease activity of the recBCD enzyme was measured to determine whether the photolabeling detects interactions between 8-N3-ATP and specific binding sites on the recB and recD subunits. Azido-ATP supported the double-stranded exonuclease activity of the recBCD enzyme, although not as effectively as ATP at low concentrations (Fig. 4), and was hydrolyzed in a DNA-dependent reaction (Fig. 5). (The apparent higher nuclelease activity at 40 μM ATP than at 200 μM (Fig. 4A) is consistent with previous observations (Eichler and Lehman, 1977).) The double-stranded exonuclease activity was reduced more than 2-fold when the recBCD enzyme and 8-N3-ATP (1 μM) were irradiated separately and then mixed to the same final concentration had the same activity as the unirradiated recBCD enzyme or enzyme that had been irradiated with or without UV irradiation, and then diluted into double-stranded exonuclease reaction mixtures containing 50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 0.67 mM DTT, 1 mg/ml bovine serum albumin, 40 μM ATP, and sonicated E. coli DNA (40 μM nucleotide, 0.020 μCi/nmol). Incubation mixtures contained: recBCD enzyme alone, no irradiation (○); recBCD enzyme alone, UV irradiation for 1 min (■); recBCD enzyme and 8-N3-ATP, no irradiation (△); recBCD enzyme and 8-N3-ATP, irradiated separately for 1 min and mixed in the double-stranded exonuclease assay (□); recBCD enzyme and 8-N3-ATP, irradiated together for 1 min (▲). RecBCD enzyme was present at 0.125 μg/ml and 8-N3-ATP at 1.4 μM in the double-stranded exonuclease reaction mixtures.

Titration of recBCD Enzyme with 8-N3-ATP—Photolabeling of the recBCD enzyme at increasing concentrations of 8-
Photolabeling saturated at high 8-N3-ATP concentrations. The recBCD enzyme and aldolase concentrations were 0.06 mg/ml, ATP was present at 50 μM (even-numbered lanes), and the [α-32P]8-N3-ATP (200 μCi/nmol) concentration was 10 (lanes 1 and 2), 20 (lanes 3 and 4), 40 (lanes 5 and 6), 75 (lanes 7 and 8), 150 (lanes 9 and 10), 250 (lanes 11 and 12), and 500 μM (lanes 13 and 14).

N2-ATP was carried out to determine whether the extent of photolabeling saturated at high 8-N2-ATP concentrations. The extent of photolabeling increased linearly with time up to 1 min of irradiation at 40 and 500 μM 8-N2-ATP (Fig. 7), indicating that the amount of enzyme labeled was directly proportional to the recBCD enzyme-8-N3-ATP complex in the solution. Scintillation counting of bands cut from the gels showed that the recB and recD subunits were labeled to only a few peptides as detected by their absorbance at 214 nm (not shown). The 32P-containing fractions were pooled, lyophilized separately, and rerun over the same column using shallower elution gradients. Radioactivity was detected in each case in many (~20) fractions. To determine whether these broad peaks represent more than one labeled peptide, the radioactive fractions were again lyophilized and analyzed by two-dimensional electrophoresis-thin layer chromatography. One major radioactive species, along with a minor one, is visible in the autoradiogram (Fig. 8, lower panels). Tryptic digestion and HPLC analysis were, therefore, performed on the isolated subunits as well as the intact enzyme.

The recBCD enzyme and aldolase were titrated with increasing concentrations of 8-N2-ATP in the presence and absence of 50 μM ATP (Fig. 8). As shown in Fig. 8A, labeling of the recB and recD subunits was saturated at high 8-N2-ATP concentrations while aldolase was not saturated at even 500 μM 8-N2-ATP. Analysis of Scatchard plots of the data (Fig. 9C) gave dissociation constants of 30 and 120 μM for binding to the recD and recB subunits, respectively. Substantial inhibition of labeling by 50 μM ATP is evident (Figs. 8 and 9B).

Some inhibition by other nucleoside triphosphates was also observed (Fig. 10); however, the extent of inhibition was substantially less than that observed with ATP, dATP, or ATPγS, showing that the sites are specific for adenine nucleotides. ADP was also inhibitory (Fig. 11), apparently binding with greater affinity to the recD than to the recB subunit.

Tryptic Peptide Mapping of recBCD Enzyme Photolabeled with [α-32P]8-N2-ATP—The high affinity of both the recB and recD subunits for 8-N2-ATP and ATP suggests that both subunits have specific binding sites for ATP. Tryptic peptide mapping of enzyme photolabeled with [α-32P]8-N2-ATP was performed to determine whether labeling is confined to only one or a few peptides as would be expected for a specific binding interaction. The recBCD enzyme (1.5 nmol) was photolabeled at 0 °C by irradiation for 1 min in the presence of [α-32P]8-N2-ATP (280 μM). The labeled protein was digested with trypsin and the peptides analyzed by HPLC. Three major radioactive peaks were observed (Fig. 12). The first corresponded to the column flow-through volume and eluted at the same position as [α-32P]8-N2-ATP. The other two radioactive peaks contained peptides as detected by their absorbance at 214 nm (not shown). The 32P-containing fractions were pooled, lyophilized separately, and rerun over the same column using shallower elution gradients. Radioactivity was detected in each case in many (~20) fractions. To determine whether these broad peaks represent more than one labeled peptide, the radioactive fractions were again lyophilized and analyzed by two-dimensional electrophoresis-thin layer chromatography. One major radioactive species, along with a minor one, is visible in the autoradiogram (Fig. 13). These results indicate that there are two peptides containing specific binding sites, consistent with photolabeling at two specific sites in the recBCD enzyme.

If the recB and recD subunits each contain specific ATP binding sites, then only a single labeled peak would be expected to appear in tryptic digests of the isolated photolabeled subunits. Tryptic digestion and HPLC analysis were, therefore, performed on the isolated subunits as well as the intact enzyme. After photolabeling, one-half of the reaction mixture was subjected to NaDodSO4-polyacrylamide gel electrophoresis, and the separated recB and recD subunits were cut out and digested with trypsin. The remainder of the mixture was treated with trypsin without separating the subunits. The HPLC elution profile of the digest of the intact enzyme (Fig. 14, top) showed three major peaks of radioactivity, as in Fig. 12. The HPLC profiles of the tryptic digests of separated photolabeled recB and recD subunits each contained a single peak of radioactivity (Fig. 14, middle and lower panels) which corresponded closely to the peaks obtained with the intact enzyme. These experiments, along with the results shown in

Fig. 8. Photolabeling of recBCD enzyme and aldolase in the presence of varying concentrations of [α-32P]8-N2-ATP and ATP. Photolabeling was carried out under standard conditions in 20 μl with 0.05 s of irradiation. The recBCD enzyme and aldolase concentrations were 0.06 mg/ml, ATP was present at 50 μM (even-numbered lanes), and the [α-32P]8-N2-ATP (200 μCi/nmol) concentration was 10 (lanes 1 and 2), 20 (lanes 3 and 4), 40 (lanes 5 and 6), 75 (lanes 7 and 8), 150 (lanes 9 and 10), 250 (lanes 11 and 12), and 500 μM (lanes 13 and 14).
FIG. 10. Inhibition of photolabeling of recBCD enzyme and aldolase by nucleotides. Photolabeling mixtures (standard conditions) contained 100 μM [γ-32P]8-N3-ATP (351 μCi/μmol) and recBCD enzyme (0.0312 mg/ml) in 35 μl. 15-μl aliquots were removed after 30 s (odd-numbered lanes) and 60 s (even-numbered lanes) of UV irradiation. Nucleotides added at 500 μM were: none (lanes 1 and 2), ATP (lanes 3 and 4), GTP (lanes 5 and 6), CTP (lanes 7 and 8), UTP (lanes 9 and 10), dATP (lanes 11 and 12), dGTP (lanes 13 and 14), dCTP (lanes 15 and 16), dTTP (lanes 17 and 18), and ATPyS (lanes 19 and 20).

Figs. 12 and 13, show that the photolabeling by 8-N3-ATP occurs largely at a single site in the recB and recD subunits, indicating that each subunit interacts specifically with the ATP analog.

The broadness of the radioactive peaks in the HPLC elution profiles could have several explanations. Covalent labeling might occur at several amino acid side chains within a single peptide, giving rise to modified peptides with slightly different mobilities. There may also be some nonspecific labeling, although this would not necessarily give rise to a single broad peak but rather to multiple peaks with different mobilities.

Interaction of Separated recBC and recD Subunits with ATP and 8-N3-ATP—Direct evidence for binding and hydrolysis of ATP by both recB and recD subunits was sought by separating the recD subunit from the recBC complex by sedimentation through glycerol gradients containing 3 M NaCl (Amundsen et al., 1986). The separation was quite efficient, although some cross-contamination remained, particularly of the recD subunit by the recBC complex, as judged by silver staining of polyacrylamide gels. No double-stranded exonuclease activity was detected in the separated subunits, although the activity could be reconstituted by incubation under conditions described by Amundsen et al. (1986). A low level of DNA-dependent ATPase was found in the fractions containing the recBC complex and a somewhat lower level in fractions containing mainly the recD subunit, which could be due to contamination with the recBC complex. Neither subunit could be photolabeled under the usual conditions. Thus, the subunits interact much more weakly with ATP when separated (i.e. recD) from recBC than in the holoenzyme.

DISCUSSION

We have shown that the recB and recD subunits of the recBCD enzyme are photolabeled by UV irradiation in the presence of 8-N3-ATP. Both polypeptides are labeled to a greater extent than the recC subunit or aldolase, a protein known to lack an ATP binding site. The photolabeling of recB and recD, therefore, represents the interaction of 8-N3-ATP with specific binding sites on the two polypeptides.

Further experiments were carried out to prove the specificity of the interaction. Titration of the recBCD enzyme with 8-N3-ATP, using irradiation times short enough that the equilibrium could be sampled without being significantly perturbed, showed that the recB and recD subunits are saturated with dissociation constants of 120 and 30 μM, respectively, while aldolase was not saturated even at 500 μM 8-N3-ATP. The saturation with relatively high affinity is to be expected for interaction of a ligand with an enzyme binding site.

The inhibition of photolabeling of both subunits by low concentrations of ATP and ADP further indicates that the sites have high affinity for ATP and other adenosine nucleotides, as well as for 8-N3-ATP. These compounds all inhibit labeling of the recD subunit to a greater extent than the recB subunit, consistent with the tighter binding of 8-N3-ATP to recD than to recB. Particularly noteworthy is the substantially greater inhibition of labeling of the recD than the recB subunit at low ADP concentrations. This fact, along with the different affinities observed for 8-N3-ATP binding, provides a strong argument against the possibility that there is only a single binding site situated at the interface of the recB and recD subunits, since in that case labeling of both subunits should be inhibited equally at a given ADP concentration. Inhibition of labeling by nonadenine-containing ribo- and deoxyribonucleoside triphosphates was somewhat weaker, again
FIG. 12. HPLC analysis of 32P-labeled peptides obtained from trypsin digestion of recBCD enzyme photolabeled with [α-32P]8-N3-ATP. RecBCD enzyme (4.4 mg/ml) was photolabeled under standard conditions (see "Experimental Procedures") in a total volume of 0.1 ml by irradiation for 1 min in the presence of 280 μM [α-32P]8-N3-ATP (290 μCi/μmol). Unincorporated label was removed by three successive 20-fold dilutions followed by centrifugation through Centricon 30 filters. The recovered protein was digested with trypsin, lyophilized, and the digest dissolved in 100 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 1% NaDodSO4. The redissolved digest was injected onto a C18 reverse-phase column (Vydac) and eluted with buffer A (0.1% (v/v) trifluoroacetic acid) for 5 min followed by a linear gradient over 90 min of 0-60% buffer B (0.1% trifluoroacetic acid (v/v) and 70% acetonitrile (v/v)) in buffer A. Fractions were collected at 0.6-min intervals and the radioactivity determined by Cerenkov counting of the entire fraction.

FIG. 13. Two-dimensional thin layer electrophoresis-chromatography of radiolabeled material obtained by HPLC separation of trypsin-digested photolabeled recBCD enzyme. Fractions containing 32P from the HPLC run shown in Fig. 12 were pooled, concentrated, and dissolved in TLC buffer I (acetic acid:formic acid:H2O, 15:5:80 (v/v/v) containing 2% methyl green). The redissolved material was spotted on Polygram CEL400 TLC plates (20 x 17.5 cm). Electrophoresis was carried out in the first dimension in buffer I at 800 V (40 V/cm). The plates were dried thoroughly, and ascending chromatography was carried out in the second dimension, eluting with butanol:pyridine:acetic acid:H2O, 32:5:25:5:20, v/v/v/v/v. The plates were then dried and autoradiographed. A, fractions 33-39 (peak 2). B, fractions 71-75 (peak 3).

emphasizing the specificity of the binding sites for adenine. No dramatic difference in affinity of one site over the other was observed for any of these nucleotides.

8-N3-ATP is hydrolyzed by recBCD enzyme in a DNA-dependent reaction. The rate observed for 8-N3-ATP hydrolysis is about one-tenth that seen at the same concentration of ATP. 8-N3-ATP also supports the double-stranded exonuclease activity of the recBCD enzyme and presumably the associated helicase activity (Muskavitch and Linn, 1982). These observations indicate that the interaction of 8-N3-ATP with at least one binding site on the enzyme is catalytically relevant. Inhibition of the double-stranded exonuclease activity when the enzyme and 8-N3-ATP are irradiated together is consistent with this conclusion.

Finally, the tryptic peptide mapping experiments shown in Figs. 12-14 support the conclusion that photoaffinity labeling occurs at a single major site within each of the recB and recD subunits.

Separation of the recD subunit from the recBC complex was carried out in an effort to determine whether interaction of the isolated subunits with ATP or 8-N3-ATP can occur. The presence of ATPase activity in the recBC complex but not recD is in agreement with results reported with the isolated subunits by Lieberman and Oishi (1974) and with the isolated recB protein (Hickson et al., 1985). The isolated recD subunit could lose its ATPase activity upon separation from the other two subunits. Alternatively, binding of ATP to the recD subunit could have a purely regulatory or effector role. The inability to photoaffinity label the isolated subunits suggests that they have reduced affinity for ATP compared to the holoenzyme. It should be noted that amino acid sequences homologous to those found in a number of ATPases are also found in the recB (Finch et al., 1986a) and recD (Finch et al., 1986b) subunits.

The standard conditions used in the photolabeling experiments (0 °C, no DNA) are not necessarily the most relevant to those in which the recBCD enzyme acts catalytically. As noted above, photolabeling does not depend on low incubation temperature but can take place at room temperature with only slightly lower efficiency. The effect of DNA is difficult
to examine because of the large amount of DNA required to provide sufficient ends for all the enzyme to bind. The UV absorbance of these solutions is so high that it is not surprising that the extent of photolabeling is greatly reduced. It is also conceivable that photolabeling by 8-N3-ATP might be at a DNA binding site which happens to have some affinity for 8-N3-ATP. This possibility seems unlikely because of the high affinity of both sites for the ATP analogue, as well as the apparent specificity for adenine nucleotides.

CONCLUSION

Our results show clearly that the recB and recD subunits of the recBCD enzyme bind 8-N3-ATP and ATP with properties expected for interactions at specific sites. The existence of two ATP binding sites raises a number of interesting questions concerning their respective roles in the catalytic activity of the enzyme. Thus, it will be of interest to determine whether ATP is hydrolyzed at each site, whether ATP binding and/or hydrolysis occur independently or whether there are interactions between the sites, and whether both sites are required for each of the multiple activities of the recBCD enzyme. Clearly, delineation of the roles of the two ATP binding sites in the recBCD enzyme will contribute much to our understanding of the mechanism of this enzyme and perhaps other helicases as well.

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REFERENCES

Abraham, K. I., and Modak, M. J. (1984) Biochemistry 23, 1176-1182
Amundsen, S. K., Taylor, A. F., Chaudhury, A. M., and Smith, G. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5558-5562
Bayley, H., and Knowles, J. R. (1977) Methods Enzymol. 46, 69-114
Bittner, M., and Vapnek, D. (1981) Gene (Amst.) 15, 319-329
Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
Davis, R. W., Botstein, D., and Roth J. R. (1980) Advanced Bacterial Genetics, pp. 116-119, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Davis, B. L., Leighton, T., and Rabinowitz, J. C. (1979) J. Biol. Chem. 254, 9220-9226
Dykstra, C. C., Palas, K. M., and Kushner, S. R. (1984) Cold Spring Harbor Symp. Quant. Biol. 49, 463-467
Eichler, D. C., and Lehman, I. R. (1977) J. Biol. Chem. 252, 499-503
Finch, P. W., Storey, A., Chapman, K. E., Brown, K., Hickson, I. D., and Emmerson, P. T. (1986a) Nucleic Acids Res. 14, 8573-8582
Finch, P. W., Storey, A., Brown, K., Hickson, I. D., and Emmerson, P. T. (1986b) Nucleic Acids Res. 14, 8583-8594
Gracy, R. W. (1977) Methods Enzymol. 47, 195-204
Haley, B. E., and Hoffman, J. F. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3367-3371
Hickson, I. D., Robson, C. N., Atkinson, K. E., Hutton, L., and Emmerson, P. T. (1985) J. Biol. Chem. 260, 1224-1229
Julin, D. A., Riddles, P. W., and Lehman, I. R. (1986) J. Biol. Chem. 261, 1025-1030
Knight, K. L., and McEntee, K. (1985) J. Biol. Chem. 260, 867-872
Kornberg, A., Scott, J. F., and Bertsch, L. L. (1978) J. Biol. Chem. 253, 3298-3304
Kowalczykowski, S. C. (1986) Biochemistry 25, 5872-5881
Laemmli, U. K. (1970) Nature 227, 680-685
Lieberman, R. P., and Oishi, M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4816-4820
Maniatis, T., Fritsch, E. F., and Samcoook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Muzavitch, K. M. T., and Linn, S. (1981) in The Enzymes (Boyer, P. D., ed) Vol. 14, pp. 233-250, Academic Press, Orlando, FL
Muskavitch, K. M. T., and Linn, S. (1982) J. Biol. Chem. 257, 2641-2648
Potter, R. L., and Haley, B. E. (1983) Methods Enzymol. 91, 613-633
Rose, L. A., and O’Connell, E. L. (1977) J. Biol. Chem. 252, 479-482
Sasaki, M., Fujishita, T., Shimada, K., and Takagi, Y. (1982) Biochem. Biophys. Res. Commun. 109, 414-422
Woody, A-Y. M., Vader, C. R., Woody, R. W., and Haley, B. E. (1984) Biochemistry 23, 2843-2848