Alteration by site-directed mutagenesis of the conserved lysine residue in the consensus ATP-binding sequence of the RecB protein of *Escherichia coli*

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ABSTRACT

The RecB and RecD subunits of the RecBCD enzyme of *Escherichia coli* contain amino acid sequences similar to a consensus mononucleotide binding motif found in a large number of other enzymes. We have constructed by site-directed mutagenesis a lysine-to-glutamine mutation in this sequence in the RecB protein. The mutant enzyme (RecB-K29Q-CD) has essentially no nuclease or ATP hydrolysis activity on double-stranded DNA, showing the importance of RecB for unwinding double-stranded DNA. However, ATP hydrolysis stimulated by single-stranded DNA is reduced by only about 5–8-fold compared to the wild-type, nuclease activity on single-stranded DNA is reduced by less than 2-fold, and the nuclease activity of the RecB-K29Q-CD enzyme requires ATP. The effects of the RecB mutation suggest that the RecD protein hydrolyzes ATP and can stimulate the RecBCD enzyme nuclease activity on single-stranded DNA.

INTRODUCTION

The RecBCD enzyme from *Escherichia coli* is one of many multisubunit protein machines in which the energy of ATP hydrolysis is coupled to alteration of the structure of DNA. The enzyme uses ATP energy for movement along DNA and for unwinding of double-stranded DNA (1). The enzyme also has a low level of nuclease activity on single-stranded DNA in the absence of ATP (2). This reaction is stimulated by ATP hydrolysis (2).

To understand the mechanism by which the RecBCD enzyme couples ATP hydrolysis to DNA unwinding and degradation requires investigation of the functions of the three subunits, RecB, RecC, and RecD. The RecB and RecD subunits are of particular interest in this regard since they have amino acid sequence homology to several other DNA helicases (3) and are therefore likely to function in the ATP-dependent movement of the enzyme along a DNA molecule, and in unwinding of double-stranded DNA (4). The RecB protein in isolation has been found to be both a DNA-dependent ATPase and a helicase (5,6). The function of the RecD subunit is unclear, since no enzymatic activities have been assigned to that protein (7,8).

One amino acid sequence shared by RecB, RecD, and other DNA helicases is a mononucleotide- or phosphate-binding motif (Gly/Ala-X-X-X-Gly-Lys-Thr/Ser, where X is any amino acid (9)). We report here the replacement by site-directed mutagenesis of the lysine residue (amino acid #29) with glutamine in the RecB protein. The resulting mutant enzyme (RecB-K29Q-CD enzyme) has very low ATP hydrolysis and ATP-dependent nuclease activity on double-stranded DNA. This is consistent with the known activities of RecB and shows that RecB is critical for DNA unwinding by the RecBCD enzyme. The RecB-K29Q-CD enzyme does have single-stranded DNA-dependent ATPase activity, and ATP-dependent nuclease activity on single-stranded DNA. These activities are reduced by only about 5–8-fold and less than 2-fold, respectively, compared to the RecBCD enzyme. The ATP-dependence of the nuclease activity suggests that the RecD subunit has ATPase activity, which has not been found in the isolated RecD protein. Similar results have recently been reported for another mutation in the same region of the RecB protein (10).

The results we find for the RecB-K29Q-CD enzyme are quite different from those we have found previously for an enzyme with the same lysine-to-glutamine mutation in the corresponding sequence in the RecD subunit (11,12,13). That enzyme (RecBCD-K177Q) has substantial double-stranded DNA-dependent ATPase, DNA helicase, and ATP-dependent nuclease activity on single- and double-stranded DNA (12). The different effects of the same mutation in RecB vs. RecD suggest that these two subunits, although both DNA-dependent ATPases, function differently in the RecBCD holoenzyme.

MATERIALS AND METHODS

Materials

ATP was purchased as a 0.1 M solution from Pharmacia. Heparin-agarose (Type I, 756 µg heparin/ml of gel) was from Sigma. Deoxyadenosine 5'-[α-³⁵S]thiotriphosphate (1000 Ci/mmol) and [γ-³²P]ATP (4500 Ci/mmol) were purchased from New England Nuclear or Amersham. Tritium-labeled plasmid and *E. coli* DNA were prepared as described (12). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were obtained

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from New England Biolabs, U.S. Biochemicals Corp., Bethesda Research Laboratories, or Promega. Restriction enzyme digestions and ligations were carried out as recommended in (14) or in protocols supplied by the manufacturers.

The cloning vector pEMBL182+ was obtained from the laboratory of Dr. David Hogness, Stanford University. This plasmid is one of the pEMBL family (15) and has the following sequence in the polylinker region:

5'-GAATTCCTCGGGTTCTAGAGCTCGAGGATCCTCTAGAGTCGACCTG-CAGGCATGCAAGCTT.

The *Xho*I recognition site is underlined. DNA sequencing reactions were done using a Sequenase kit (U. S. Biochemicals Corp.). DNA sequencing primers were prepared by Dr. Tomas Kempe in the Protein and Nucleic Acids Laboratory, University of Maryland.

RecB mutagenesis

RecB gene subcloning. The plasmid pDJ02 (11), containing a 13.5 kilo-base pair (kb) *Sma*I-*Bam*HI fragment encoding the *recBCD* genes, was digested with *Xho*I to produce a 3166 base-pair (bp) fragment (nucleotides #7,947–11,112 according to the numbering in (16,17)) containing part of the *recB* gene, including the site to be mutagenized. The 3166 bp fragment was isolated from a 1% agarose gel by adsorption to DE81 paper (18). The isolated fragment was joined to *Xho*I-cleaved, calf intestinal phosphatase-treated pEMBL182+ vector DNA with T4 DNA ligase. The ligation mixture was used to transform *E. coli* strain HB101 to carbenicillin resistance. Plasmid DNA was isolated from several colonies (ref. # 14, p. 368) and digested with *Xho*I. Several were found with the 3166 bp fragment ligated to pEMBL182+ and were called pBEM182.

Site-directed mutagenesis. The mutagenic primer, 5'-GCACA GGCCAAACCTTTACG, where the underlined C changes an AAA lysine codon to a CAA codon encoding glutamine, was purchased from Amber, Inc., Ridgefield, CT. The nucleotide residue changed is #9051, and the lysine residue is amino acid #29 in the RecB sequence. pBEM182 was transformed into *E. coli* strain JM109 for preparation of the single-stranded form of the plasmid. Single-stranded DNA was prepared using the helper phage M13K07 following the procedure in the MutaGene kit from BioRad. The mutagenesis was done by the phosphorothioate method (19) and was carried out using the enzymes and reagents obtained from Amersham.

The mutagenesis mixture was transformed into *E. coli* strain TG1 and plasmid DNA was isolated from several transformants. Potential mutants were detected by digesting with restriction endonuclease *Hae*III. The desired mutation introduces a new *Hae*III recognition site (GGCC) into pBEM182. There are 26 *Hae*III sites in pBEM182 and the largest fragment is 685 bp. The new site is found within this largest fragment, and results in its being cleaved to 404 and 281 bp fragments. *Hae*III digests were examined by electrophoresis on 5% polyacrylamide gels. Plasmids whose *Hae*III digests lacked the 685 bp fragment but showed the two new smaller fragments were then used for DNA sequencing reactions with a primer which binds 85 nucleotides upstream of the mutagenized site. Plasmid DNA to be sequenced was prepared by the acid-phenol procedure (20). Plasmids containing the A–C mutation were named pBEM-BK29Q.

Reconstruction of plasmids containing the mutagenized *recB* gene. The wild-type *Xho*I fragment in the plasmid pFS11-04 (21) was replaced by the mutagenized *recB* gene fragment as follows. pBEM-BK29Q was transformed into JM109 and re-isolated. The DNA was digested with *Xho*I and the 3166 bp fragment was isolated from a 0.9% agarose gel by the GeneClean procedure (BIO101 Inc., La Jolla, CA). pFS11-04 was also digested with *Xho*I, purified by phenol/chloroform extraction and ethanol precipitation, and treated with T4 DNA ligase to recircularize the DNA. The ligation mixtures were transformed into *E. coli* strain HB101. Plasmids were isolated from several transformants and digested with *Xho*I to identify those which had circularized with the exclusion of the original wild-type 3166 bp fragment. This plasmid (pFS-X) was then digested with *Xho*I and ligated to the isolated mutagenized *recB* fragment, to reconstruct the original pFS11-04, except now containing the mutation in the *recB* gene. The orientation of the inserted fragment was checked by digesting the resulting plasmid (pFS-BK29Q) with *Pst*I. pFS-BK29Q was used to express the mutant *recB* gene and the wild-type *recC* and *recD* genes in the same cell.

Two control plasmids were also prepared, to test the possibility of undesired mutations at other sites. For the first (pFS11-04'), the original unmutagenized *Xho*I fragment in pBEM182 was ligated back to pFS-X. The second was constructed as follows. The DNA sequence in pBEM-BK29Q corresponding to nucleotides #8845 to 9149, spanning the start of the *recB* coding region (#8967) to a *Bst*EII recognition site (#9149) and including the mutagenesis site, was determined in its entirety and no changes or ambiguities were found, other than the site-directed A–C change. The 1203 bp *Xho*I-*Bst*EII fragment in pBEM-BK29Q (nucleotides #7946 to 9149) was then replaced with an unmutagenized fragment from pBEM182. This new, complete 3166 bp *Xho*I fragment was then transferred to pFS-X, as above, to give pFS-BK29K. The chimeric 3166 bp *Xho*I fragment contains 1203 bp (*Xho*I to *Bst*EII) of wild-type, unmutagenized DNA (including the unaltered lys₂₉ codon), and 963 bp (*Bst*EII to *Xho*I) of DNA originally subcloned into pBEM182 and carried through the mutagenesis procedure, but which should have been unchanged by that procedure.

Table I. Nuclease activity in crude cell extracts^a.

plasmid	genotype	[protein]	nuclease activity ^b	
			–ATP	+40 μM ATP
pFS11-04 ^c	wild-type	0.4 mg/ml	125	636
		0.26	138	741
pFS-BK29Q ^c	<i>recB-K29Q</i>	0.96	103	102
		0.96	84	102
pFS11-04 ^c	unmutagenized <i>Xho</i> I fragment	1.12	77	280
		0.94	74	227
pFS-BK29K ^c	wild-type			
	<i>Xho</i> I- <i>Bst</i> EII	1.08	72	298
	mutant <i>Bst</i> EII- <i>Xho</i> I	0.88	82	293
pDJ05 ^d	wild-type	0.98	48	898
pDJ05-BK29Q ^d	<i>recB-K29Q</i>	0.945	32	41
		1.16	36	40

^a Nuclease reaction mixtures contained 50 mM TrisHCl, pH 8.5, 10 mM MgCl₂, 0.67 mM DTT, and *E. coli* [³H]DNA (40 μM nucleotides). Cell extracts were prepared and acid-soluble DNA fragments were measured as in (11).

^b Nuclease activity is given as (nmol acid-soluble DNA)/(mg extract protein)/10 min assay.

^c The host strain was V186 (*ΔrecBCD*).

^d The host strain was JC5519 (*recB21 recC22*).

DNA sequencing. The mutagenized *Xho*I fragment in pBEM-BK29Q was sequenced using primers synthesized to bind 200–300 nucleotides apart on the single-stranded form of the plasmid. Several reactions were done for each region to resolve ambiguities. No base changes were found except for the site-directed one. Four positions far from some primers were ambiguous, leading us to construct the control plasmids described above.

Expression and purification of the RecB-K29Q-CD enzyme

The plasmids pFS11-04, pFS-BK29Q, pFS11-04', and pFS-BK29K were transformed into V186 (Δ *recBCD* (22)) for expression of the enzymes. The nuclease activity was determined by measuring production of acid-soluble [3 H]DNA fragments using reaction conditions as in (11). The substrate for measurements in crude cell extracts and during the purification was native or denatured *E. coli* [3 H]DNA.

The RecB-K29Q-CD enzyme was purified from an 18 liter culture of V186[pFS-BK29Q] in LB broth containing ampicillin (50 mg/l) as described for the wild-type enzyme (11). The protein concentrations in the purification fractions, and of the final purified enzyme, were determined by the Bradford method (23) using bovine serum albumin (BSA) as the standard.

Nuclease reaction measurements

Reaction mixtures with the purified enzyme contained 50 mM TrisHCl, pH 7.5, 10 mM MgCl₂, and 0.67 mM dithiothreitol (DTT). The substrate for the purified enzyme was pTZ19R [3 H]DNA (2863 bp) cleaved with *Sma*I. Denatured DNA was prepared by immersing the double-stranded DNA in boiling water for 5 min and then placing the tube in ice water.

ATP hydrolysis

ATP hydrolysis was measured by thin layer chromatography on polyethyleneimine plates (Sigma) using [γ - 32 P]ATP as described

(12). The DNA substrate was unlabeled pTZ19R DNA cleaved with *Sma*I, and the reaction conditions were the same as for the nuclease reactions with the purified enzyme.

RESULTS

Activities in crude cell extracts

We first tested the enzymatic activity encoded by the pFS plasmids in crude cell extracts. Single colonies of V186 transformed with each plasmid were grown overnight in LB medium containing ampicillin (50 μ g/ml) and thymidine (50 μ g/ml). The cells were harvested, lysed, and the ATP-dependent nuclease activity on double-stranded [3 H]DNA was measured as in (11). Table I shows the nuclease activity on double-stranded DNA found in each cell extract. There is a significant amount of ATP-stimulated nuclease activity on double-stranded DNA in crude extracts of cells expressing the wild-type genes encoded by pFS11-04. The nuclease activity with the mutant plasmid pFS-BK29Q is very close to the background reaction observed with no ATP. The mutation has therefore caused a substantial reduction in the ATP-dependent nuclease activity of RecBCD with double-stranded DNA. The control plasmids, particularly pFS-BK29K, show that the reduction in nuclease activity is due to the site-directed mutation and not some other, unknown, mutation. Thus, replacement of the mutagenized 1203 bp *Xho*I-*Bst*EII fragment (182 bp of the *recB* gene) with the original, wild-type fragment restored substantial ATP-dependent nuclease activity. The DNA sequencing (see 'Materials and Methods') provides additional support for this conclusion.

We also inserted the mutagenized *recB* gene fragment into the plasmid pDJ05, which we have used previously to prepare the wild-type RecBCD enzyme and the mutant RecBCD-K177Q enzyme (11). However, we were unable to maintain this plasmid in cultures of V186. Colonies of V186 transformed with pDJ05-BK29Q were obtained on plates, but they did not grow

Figure 1. A. Exonuclease activity on single-stranded DNA eluted from DEAE-cellulose. Ammonium sulfate (0.282 g/ml) was added to the crude lysate of V186[pFS-BK29Q] and the precipitated protein was run on a DEAE-cellulose chromatography column. The bound protein was eluted in a gradient of 0.15 to 0.6 M NH₄Cl. Column fractions were analyzed for total protein (■) and exonuclease activity on heat-denatured *E. coli* [3 H]DNA in the absence (○) and presence (●) of 0.2 mM ATP. B. Analysis of column fractions by SDS-polyacrylamide gel electrophoresis. Samples (50 μ l) from the indicated column fractions were denatured by boiling for 3 min in SDS-gel loading dye (0.03 M TrisHCl, pH 6.8, 0.6% SDS, 80 mM 2-mercaptoethanol, 6% glycerol, and 0.002% bromophenol blue). The samples were then run on a 7.5% polyacrylamide gel containing SDS. The gel was stained after the run in Coomassie Brilliant Blue R-250. Purified RecBCD enzyme was included as a marker.

Figure 2. Purified RecB-K29Q-CD enzyme. RecB-K29Q-CD enzyme obtained from the final step in the purification procedure (heparin-agarose column chromatography) was run on a 7.5% polyacrylamide gel containing SDS. Lane 1: RecB-K29Q-CD enzyme (2.6 μ g total protein); Lane 2: Purified RecBCD enzyme (2.2 μ g).

Figure 3. Exonuclease activity of the RecB-K29Q-CD enzyme. Reaction mixtures contained 50 mM TrisHCl, pH 7.5, 10 mM MgCl₂, 0.67 mM DTT, and 20 μ M (nucleotides) *Sma*I-cut pTZ19R [³H]DNA. A. Double-stranded DNA. (●) 40 μ M ATP, 0.16 nM RecBCD enzyme; (■) 1 mM ATP, 0.16 nM RecBCD enzyme; (□) 1 mM ATP, 1.8 nM RecB-K29Q-CD enzyme. B. Denatured DNA, 200 μ M ATP. (●) 0.39 nM RecBCD enzyme; (○) 0.36 nM RecB-K29Q-CD enzyme; (■) 0.73 nM RecB-K29Q-CD enzyme, no ATP.

well in liquid culture. Therefore, we used JC5519 (*recB21 recC22*) as the host. Table I also shows the nuclease activity observed in extracts of JC5519 transformed with the pDJ05 plasmids.

Purification of the RecB-K29Q-CD enzyme

We proceeded to attempt to purify the mutant RecB-K29Q-CD enzyme, to see whether it retains any of the activities of the wild-type enzyme. We followed the procedure we used previously for the wild-type and RecBCD-K177Q enzymes (11). The first chromatography column in this procedure is DEAE-cellulose.

A peak of protein was eluted from this column in a gradient of NH₄Cl. ATP-independent exonuclease activity on single-stranded DNA was found in fractions *ca.* 60–90, while fractions *ca.* 80–105 have nuclease activity on single-stranded DNA which is stimulated by ATP (200 μ M) (Fig. 1A). We detected no ATP-dependent nuclease activity on double-stranded DNA in the fractions eluted from the DEAE-cellulose column. The RecB, RecC, and RecD proteins were visible in fractions *ca.* 75–105 when samples were analyzed on a 7.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS) (Fig. 1B). This result suggests that the mutant enzyme does have ATP-dependent nuclease activity on single-stranded DNA, as does the wild-type enzyme. Fractions 80–102 were pooled and carried through the purification procedure.

The final preparation obtained after chromatography on hydroxylapatite and heparin-agarose contained the RecB-K29Q, RecC, and RecD proteins, and a single major contaminant (Fig. 2). Analysis of the gel shown in Fig. 2 by densitometry showed that the contaminant was about 70% of the total protein in the preparation. The protein concentration in this sample, determined by the Bradford method using BSA as a standard, was about 0.044 mg/ml. If the RecB-K29Q-CD enzyme is 30% of the total, then its concentration is about 0.0132 mg/ml, or about 40 nM, using a molecular mass of 330,000 for the RecB-K29Q-CD enzyme. This value is approximate given the low protein concentration and the presence of the contaminant, but it provides a number which allows comparisons to be made between the mutant and wild-type enzymes.

Efforts are underway to obtain more purified RecB-K29Q-CD enzyme so that detailed kinetics studies can be done. Initial attempts using chromatography on phosphocellulose and ATP-agarose were unsuccessful.

Nuclease activity of the purified RecB-K29Q-CD enzyme

The nuclease activities of the wild-type RecBCD enzyme and the RecB-K29Q-CD enzyme are shown in Fig. 3 and Table II. The mutant has virtually no detectable nuclease activity on double-stranded DNA (Fig. 3A). However, the mutant has nuclease activity on single-stranded DNA which is very close to that of the wild-type (Fig. 3B). This activity requires ATP (Fig. 3B). The *ATP-dependence* of the nuclease activity on single-stranded DNA strongly indicates that the reaction is catalyzed by the RecB-K29Q-CD enzyme and not by a contaminant in the preparation. The relative rates with 20 and 200 μ M ATP are very similar to those for the wild-type enzyme (Table II), suggesting that the *K_m* for ATP in the nuclease reaction has not been substantially altered by the mutation.

ATP hydrolysis by the purified RecB-K29Q-CD enzyme

The ATP hydrolysis activity of the two enzymes is compared in Fig. 4 and Table II. The mutant has very low ATPase activity with blunt-ended double-stranded DNA (Fig. 4A). The activity of the mutant is reduced by about 500–800-fold compared to the wild type (Table II). The mutant enzyme has greater ATPase activity in the presence of denatured DNA than it does with double-stranded DNA (Fig. 4B, Table II). This is the opposite of the wild type, which has greater activity with double- than with single-stranded DNA. The single-stranded DNA-dependent ATPase activity of the mutant is reduced by about 5-fold compared to the wild type, at 20 μ M ATP, and by about 8-fold at 200 μ M ATP. A ten-fold increase in the ATP concentration (20 to 200 μ M) brought about less than a two-fold change in the

Table II. ATPase and nuclease activities of the RecBCD and RecB-K29Q-CD enzymes.

DNA	[ATP] (μ M)	reaction rate/[enzyme] (min^{-1})	
		Exonuclease ^a RecBCD	RecB-K29Q-CD
ss ^b	20	590 ($\pm$ 13)	520 ($\pm$ 20)
	200	1037 ($\pm$ 76)	720 ($\pm$ 20)
ds ^c	40	$\geq$ 13,000	2
	1000	$\geq$ 10,000	13
ATP Hydrolysis ^d			
ss	20	1200 ($\pm$ 250)	255 ($\pm$ 30)
	200	3480 ($\pm$ 40)	440 ($\pm$ 65)
ds	20	14,400 ($\pm$ 1400)	30 ($\pm$ 2)
	200	80,000 ($\pm$ 2000)	105 ($\pm$ 38)

^a Reaction mixtures contained 50 mM TrisHCl, pH 7.5, 10 mM MgCl₂, 0.67 mM DTT, *Sma*I-cut pTZ19R [³H]DNA (20 μ M nucleotides), and the indicated ATP concentration.

^b Reaction mixtures contained 0.195 or 0.391 nM RecBCD enzyme, and 0.364 or 0.727 nM RecB-K29Q-CD enzyme.

^c Reaction mixtures contained 0.16 nM RecBCD enzyme or 0.18 nM RecB-K29Q-CD enzyme. The rates are approximate due to the non-linearity of the reaction with the RecBCD enzyme, and the slow rate of the RecB-K29Q-CD enzyme-catalyzed reaction (see Fig. 3A).

^d Reaction mixtures were the same as the nuclease mixtures except the DNA was non-radioactive, *Sma*I-cut pTZ19R DNA (100 μ M nucleotides), the ATP was [γ -³²P]ATP, and the enzymes were at 0.17 nM (RecBCD enzyme) or 2 nM (RecB-K29Q-CD enzyme). ATP hydrolysis was measured by thin layer chromatography as in (12).

Figure 4. DNA-dependent ATPase activity of the RecB-K29Q-CD enzyme. Reaction mixtures were as in Fig. 3, except they contained 200 μ M [γ -³²P]ATP, and the DNA substrate was 100 μ M (nucleotides) *Sma*I-cut pTZ19R DNA. A. Double-stranded DNA. (●) 0.16 nM RecBCD enzyme; (○) 2 nM RecB-K29Q-CD enzyme. B. Denatured DNA. (●) 0.16 nM RecBCD enzyme; (○) 2 nM RecB-K29Q-CD enzyme; (■) 2 nM RecB-K29Q-CD enzyme, no DNA.

ATP hydrolysis rate, indicating that the mutation has not simply caused an increase in the K_m for ATP in the ATPase active site of RecB. The mutant enzyme has essentially no ATPase activity in the absence of DNA (Fig. 4B).

DISCUSSION

The RecB-K29Q-CD enzyme we have prepared in this work gives several pieces of important information about the mechanism of the RecBCD enzyme. Of particular interest is comparison of the enzymatic activities of the RecB-K29Q-CD enzyme to those we have reported previously for the RecBCD-K177Q enzyme (11,12,13). The latter has a lysine-to-glutamine change in the same consensus ATP-binding sequence in the RecD subunit as is found in the RecB subunit.

The RecB protein in isolation is a DNA-dependent ATPase with either single- or double-stranded DNA, and has helicase activity (5,6). The fact that the mutation in RecB causes such a large reduction in ATP hydrolysis with double-stranded DNA is thus consistent with the known activities of RecB. We have not yet tested the RecB-K29Q-CD enzyme for helicase activity. However, the low level of ATP hydrolysis with double-stranded DNA makes it very unlikely that RecB-K29Q-CD has any substantial helicase activity. The wild-type enzyme hydrolyzes more than one ATP molecule for every DNA base pair it unwinds (4). Therefore a reduction in ATP hydrolysis of the magnitude seen in RecB-K29Q-CD compared to RecBCD should cause at least an equivalent reduction in helicase activity.

The retention of ATPase activity with single-stranded DNA in the RecB-K29Q-CD enzyme is therefore quite interesting. There are two possible explanations for this finding. One, which we feel is less likely, is that the mutant RecB-K29Q subunit lacks ATP hydrolysis activity with double-stranded DNA but remains a single-stranded DNA-dependent ATPase. The mutation could have affected binding to double-stranded DNA or the helicase activity, but not ATP hydrolysis directly. However, the mutation is within the sequence in RecB which corresponds to the ATP-binding site. This sequence is found in many other proteins which bind mononucleotides or phosphorylated substrates (9). The three-dimensional structures of at least four of these proteins have been determined (*E. coli* RecA protein (24), the translation elongation factor Tu (25), the *ras* p21 protein (26), and adenylate kinase (27,28)). Substrate or product analogues (i. e., ADP, etc.) are bound in each protein at this site with a phosphate residue near the lysine side chain. It is therefore reasonable to conclude that the sequence in RecB is part of the ATP binding site. The mutation should affect ATP binding to the RecB protein, regardless of the type of DNA substrate, and affect the helicase reaction indirectly because of reduced ATP hydrolysis.

A more likely explanation for the single-stranded DNA-dependent ATP hydrolysis activity of RecB-K29Q-CD is that this reaction is catalyzed by a subunit other than RecB, most likely the RecD subunit. Photoaffinity-labeling experiments showed that the RecD protein binds ATP in the holoenzyme (29). The RecD protein has amino acid sequence homology in several short stretches to the RecB protein and to a number of DNA helicases (3). Included are two amino acid sequences thought to be involved in ATP binding (30): the 'A'-sequence in which we have performed the mutagenesis, and the 'B'-sequence consisting of four hydrophobic residues (leu, i-leu, val, or met) followed by asp. The RecD protein in isolation has neither ATPase nor nuclease activity (7,8), but the overexpressed, isolated RecD

protein forms aggregates (7), and so activity measurements are equivocal. It is also possible that the RecD protein requires the other subunits in order to attain its active conformation. Moreover, an active site in RecD could be shared with another subunit. If this were the case, then RecD by itself would have very low activity (i. e., ATPase), but the activity would be present in the RecB-K29Q-CD enzyme if the shared site is in RecC or in an unaltered region of RecB.

The nuclease results are in agreement with this conclusion and shed further light on the functions of the RecB and RecD subunits. The low level of ATP-dependent nuclease activity on double-stranded DNA in the RecB-K29Q-CD enzyme is consistent with the mutation having affected DNA unwinding, since degradation of double-stranded DNA requires its concomitant unwinding (31,32). The presence of nuclease activity on single-stranded DNA shows that the mutation has not disrupted the nuclease active site itself. The ATP-dependence of this nuclease activity suggests that ATP hydrolysis by the RecD protein can stimulate DNA cleavage catalyzed by the RecB-K29Q-CD enzyme.

It is interesting that the reduction in single-stranded DNA-dependent ATP hydrolysis is greater than that in the single-strand nuclease activity (Table II). If ATP hydrolysis by the wild-type enzyme occurs at both the RecB and RecD subunits, while that by RecB-K29Q-CD is primarily, or exclusively, catalyzed by the RecD subunit, then this result can be explained. Some single-stranded DNA-dependent ATP hydrolysis catalyzed by the RecBCD enzyme may not lead to nuclease cleavage. There is thus a high ratio of ATP hydrolysis to acid-soluble DNA production. In the mutant, if only one subunit hydrolyzes ATP but the nuclease active site is unimpaired, then there might be less ATP hydrolyzed independently of DNA cleavage. Further, it could be that ATP hydrolyzed by RecD is mainly responsible for stimulating the nuclease activity on single-stranded DNA of RecBCD. The observation that the RecD mutant enzyme (RecBCD-K177Q enzyme) has reduced, but not nonexistent, ATP-dependent nuclease activity on single-stranded DNA suggests however that ATP hydrolyzed by RecB can stimulate nuclease activity (12). The apparent ratio of ATP hydrolyzed per acid-soluble nucleotide produced by the RecB-K29Q-CD enzyme is about 1 ATP for 2 soluble nucleotides (Table II). The significance of this ratio is unclear, as the reaction conditions, particularly the DNA concentrations, were different in the two measurements. The nuclease measurement is also somewhat ambiguous, since a single nuclease cleavage reaction will produce more than one soluble nucleotide residue (2).

The effects of the K29Q mutation in RecB are quite different from the effects of the corresponding mutation (K177Q) in RecD (11,12,13). While the RecB-K29Q-CD enzyme is quite deficient in double-stranded DNA-dependent activities (nuclease, ATPase, and, most likely, helicase), the RecBCD-K177Q enzyme retains all these activities (12). Each is reduced quantitatively compared to the wild-type in the RecBCD-K177Q enzyme, and there are qualitative changes as well. Nonetheless, the RecBCD-K177Q enzyme is an active and processive DNA helicase capable of unwinding at least 1000 bp before dissociating from the DNA substrate (13). This enzyme is also reduced in its ATP-dependent nuclease activity on single-stranded DNA (12). These results show that the RecD subunit has a role in DNA unwinding, although it is not absolutely required, and its precise function remains unclear. The RecBCD-K177Q enzyme is also similar in its ATP-dependent activities to the RecBC enzyme, which completely lacks RecD (33). We have suggested that the RecD

and RecB subunits may alternate in ATP hydrolysis on double-stranded DNA, based on the steady-state kinetics of ATP hydrolysis (33).

The present results with the RecB-K29Q-CD enzyme indicate that the RecD subunit does hydrolyze ATP. However, this subunit may be a *single-stranded* DNA-dependent ATPase only. The results suggest that DNA unwinding is initiated by the RecB subunit, and that the RecD subunit binds to unwound DNA produced by RecB. This binding stimulates ATP hydrolysis catalyzed by RecD. ATP hydrolysis by RecD could have several functions. RecD could participate directly in the DNA unwinding reaction (4), or ATP hydrolysis by RecD could enable that subunit to move along the unwound DNA strand produced by RecB. The function of the subunit could then be to maintain tight binding of the RecBCD enzyme to the DNA, and thereby allow rapid and highly processive (34) catalysis by RecBCD. This is consistent with the reduced rate and processivity of both the RecBCD-K177Q (13) and RecBC enzymes (33).

The RecD subunit bound to the unwound DNA strand also seems to be important for the nuclease reaction. The low level of nuclease in the RecBC enzyme (33,35) compared to the RecBCD and RecBCD-K177Q enzymes is consistent with participation of RecD in the nuclease reaction. The subunit location of the nuclease active site in the RecBCD enzyme, the stringency of coupling between ATP hydrolysis and DNA cleavage, and the mechanism of that coupling are not known. The nuclease cleavage reaction itself is likely to be independent of ATP hydrolysis, since the RecBCD enzyme has ATP-independent nuclease activity on single-stranded DNA (2). DNA binding and ATP hydrolysis by RecD could stimulate single-strand nuclease activity indirectly by affecting substrate binding affinity or the rate of dissociation of the enzyme from the cleaved product.

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