Purification and Properties of the RecR Protein from Bacillus subtilis 168*

Juan C. Alonso, Asita C. Stiege, Beate Dobrinski, and Rudi Lurz
From the Max-Planck-Institut für molekulare Genetik, Ihnestraße 73, D-1000 Berlin 33, Germany

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Genetic evidence suggests that the Bacillus subtilis recR gene product is involved in DNA repair and recombination. To assign a biochemical function to the recR gene product, the RecR protein was labeled and purified by monitoring the radioactive label.

NH2-terminal protein sequence analysis of RecR was consistent with the deduced amino acid sequence of the recR gene. The RecR protein (molecular mass of 25 kDa, isoelectric point 5.4) bound single- and double-stranded DNA in a filter binding assay. RecR-DNA complex formation is enhanced by the presence of a damage in the DNA substrate. The RecR-DNA complex formation proceeds in the absence of divalent cations and nucleotide cofactors, but is markedly stimulated by ATP and divalent cations. In our experimental conditions the apparent equilibrium constants of the optimized RecR-DNA complexes are 3 \times 10^{-7} \text{ M} and 9 \times 10^{-7} \text{ M} for damaged and undamaged DNA, respectively. The binding reaction is cooperative. Electron microscopy studies show that the presence of divalent cations increases the rate of RecR protein self-assembly. Addition of ATP or dATP promotes the organization of discrete series of quaternary structures on DNA, but ATP\gamma\S inhibits the DNA binding activity. A possible mechanism for the RecR function in DNA repair is discussed.

Cells have evolved several distinct mechanisms to help them to maintain the structural and informational fidelity of their DNA. In bacteria, multiple pathways for DNA repair and homologous DNA recombination can account for the fidelity of the genetic information. A genetic analysis of the recombinational mechanisms in Bacillus subtilis shows that it shares similarities and also shows differences with the recombinational pathways of Escherichia coli.

Homologous recombination in wild-type E. coli occurs primarily through the RecBCD pathway (counterpart of B. subtilis \(\beta\) pathway), whereas in B. subtilis postreplication repair and homologous recombination occur mainly through the \(\alpha\) pathway (counterpart of E. coli RecF pathway) (1–3). Genetic analysis shows that the RecBCD or \(\beta\) pathway is dependent on the RecA, RecBCD (exonuclease V, termed also AddAB(C?) in B. subtilis) and single-stranded DNA-binding proteins (SSB)1 (1–5). At present, the existence of a third subunit (AddC?) in the B. subtilis exonuclease V enzyme is controversial (5, 6).

The biochemical activities of the products required for the RecBCD or \(\beta\) pathway are well characterized. The RecBCD(AddAB(C?)) enzyme commencing at dsDNA ends unwinds the DNA. Upon encountering a properly oriented cis-acting DNA sequence termed Chi site, the RecBCD enzyme nicks the DNA and generates an invasive ssDNA tail. This Chi-dependent invading strand, which must contain homology to the recipient supercoiled DNA substrate, generates a 3'-end that is the substrate for DNA strand transfer promoted by RecA and SSB (for reviews, see Refs. 7–10).

What is known about the RecF or \(\alpha\) pathway of homologous recombination? In both bacteria, mutations in the recF, recR, or recL (phenotypical counterpart of E. coli recO) genes were shown either to inhibit or reduce a variety of DNA metabolic events, e.g. SOS induction, DNA recombination, and postreplication repair (1, 3, 11–16). Based on their phenotype and on shared indirect suppressors the recR, recF, and recO(recL) functions were placed within the same epistatic group (2, 3, 17, 18). In both E. coli and B. subtilis, the recR, recF, and recO(recL) genes are part of the RecF and \(\alpha\) recombination pathways, respectively (1–3, 12, 13). Strains carrying mutations in any one of these three genes are virtually identical in phenotype. Therefore, it has been postulated that the E. coli RecF, RecO, and RecR could form a functional protein, termed RecPOR. The same could hold true for B. subtilis (RecFLR) (1–5, 17, 18).

Unlike the RecBCD or \(\beta\) pathway, the RecF or \(\alpha\) pathway appears to utilize a DNA substrate without dsDNA ends. Although the genetic studies have demonstrated the involvement of the RecA, RecR, RecF, RecO(RecL), and SSB products in the RecF or \(\alpha\) pathway of recombination (1, 12, 13, 17), the biochemical mechanism of this process and the substrate recognition have yet to be elucidated.

Recently, it has been shown by filter binding and gel filtration assays, that the E. coli RecF protein binds to ssDNA (19, 20). Competition binding experiments with different DNA molecules demonstrated that RecF binds preferentially to linear ssDNA molecules (19). Furthermore, it was proposed that the RecF proteins of different origin have an associated ATPase activity (21). The role of the RecL(RecO) and RecR proteins is unknown (22, 15). To investigate the biochemical properties of the B. subtilis RecR protein (previously termed RecM protein, 17) we overproduced and highly purified the protein. We show that the binding of the RecR protein to dsDNA is enhanced when the DNA substrate is pretreated with different DNA damaging agents. The results presented provide the first evidence that a protein involved in postreplication repair and homologous DNA recombination between two circular molecules distinguishes between damaged and undamaged DNA.

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1 The abbreviations used are: SSB, single-stranded DNA-binding protein(s); adDNA, single-stranded DNA; dsDNA, double-stranded DNA; DTE, dithioerythritol; DTT, dithiothreitol; TEA, triethanolamine; ATP\gamma\S, adenosine 5'-O-(thiotriphosphate).
ExPERIMENTAL PROCEDURES

**Bacterial Strain and Plasmids**—E. coli strain BL21(DE3), used as a host strain for RecR overproduction, was a gift of Dr. William Studier. Plasmids pT181 (24), pUC18 (25), pBT56 (22), pLysE (23), pBT141, pBT142, and pBT143 (26) have been previously described. Plasmids pBT142 and pBT143 have one or two base pair insertions with respect to pBT141.

**Enzymes and Reagents**—Protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin, and leupeptin) were from Boehringer Mannheim. DTE and iso-propl-1-thio-β-galactoside were from Sigma. Cellulose phosphate was from Whatman. Heparin-Sepharose LC-6B and Sepharose 4B-CL were from Pharmacia.

All chemicals used were reagent-grade, and solutions were made in quartz-distilled water. ATP, GTP, ADP, and TTP were purchased from Boehringer Mannheim. Concentrations of adenine nucleotides were determined spectrophotometrically by using an extinction coefficient of 1.54 × 10^4 M^-1 cm^-1 at 260 nm. They were dissolved as concentrated stock solutions at pH 7.0, unless stated otherwise. ATP·S contained less than 10% ADP and here was used without additional purification.

^2H]PhATP, ^2H]methionine, and [methyl-^3H]histidine were from Amersham Corp. Ultrapure acrylamide was from Serva. The low molecular weight protein marker was obtained from Gibco/BRL.

Methyl methanesulfonate was purchased from Eastman Kodak. Ethyl methanesulfonate was obtained from TCI, Tokyo, Japan. Mitomycin C, Mitomycin C-1-oxide, and hydroxymethyl hydroxyidine were purchased from Sigma.

**DNA and Protein Labeling**—Covalently closed circular plasmid DNA was purified by using the Sds lysis method (27) followed by purification on a cesium chloride-ethidium bromide gradient. End-labeling of linear dDNA was performed by filling in the restriction site with the large fragment of DNA polymerase I.

The RecR protein was specifically labeled with ^3H]methionine as previously described (22).

**RecR Protein Purification**—A culture (1 liter) of E. coli BL21(DE3) strain containing pBT56 and pLysE was grown in L medium (27) and induced as described by Alonso et al. (22). The cells were harvested by centrifugation at 4 °C and mixed with a similar cell lysate containing RecR protein labeled with ^3H]methionine. The pellet was quickly frozen and stored at -20 °C. The cell paste was resuspended in 20 ml buffer A (25 mM HEPES-KOH, pH 7.0, 500 mM KCl, 2 mM ZnSO4, 15% sucrose), lysozyme was added to a final concentration of 1 mg/ml, and incubated on ice-water for another 30 min. The cells were then frozen in a dry ice-ethanol bath and thawed in a 37 °C bath. This freeze-thaw procedure was repeated twice. After cell lysis we observed that in the absence of 1 mM Zn²⁺ the putative RecR (25 kDa) and in the RecR* (19 kDa) polypeptide. Immunoblot analysis was used to confirm that these polypeptides were synthesized from the recR gene.

The RecR protein was readily sedimented by low speed centrifugation, whereas the 19-kDa product was soluble (Fig. 1A, lane 3). Therefore, the extract was precipitated by centrifugation at 27,000 × g for 30 min. The pellet (fraction 2) was diluted 10 times in buffer B (25 mM HEPES-KOH, pH 7.0, 500 mM KCl, 1 mM DTE) and centrifuged at 12,000 × g for 30 min. The pellet (fraction 1) was collected and resuspended in 20 ml of buffer C (25 mM HEPES-KOH, pH 7.0, 100 mM KCl, 1 mM DTE, 1 mM EDTA) containing 2 mM deionized urea. The solution was then incubated at 15 °C in 4 °C for 15 min and centrifuged at 12,000 × g for 30 min. The supernatant was retained (fraction 4). The addition of urea, which was used throughout the purification scheme, solubilizes the RecR protein. Fraction 4 was loaded onto a phosphocellulose column equilibrated with buffer C containing 2 M urea. The void volume of the above column was loaded onto a heparin-Sepharose column. The column was washed with 10 column volumes of buffer D (25 mM HEPES-KOH, pH 7.0, 1 mM DTE, 1 mM EDTA, 2 M urea) containing 200 mM KCl and eluted by using a step gradient from 200 to 600 mM KCl in buffer D. The fractions corresponding to the radioactive material, which coincides with the pure RecR protein, were pooled. Refolding conditions were chosen to minimize formation of aggregates. In short, urea was slowly removed by dialyzing against equal volumes of buffer E (25 mM HEPES-KOH, pH 7.0, 50 mM KCl, 1 mM DTE, 0.1 mM EDTA, 1 M urea) at 4 °C for 4 h. After removal of urea, the RecR protein was dialyzed overnight against buffer E containing 20% glycerol. Samples were stored at -20 °C.

**Filter Binding Assay**—The formation of RecR-DNA complexes was measured by using alkali-treated filters (Millipore, type HAWP 45 µm) as described by McEntee et al. (28). Standard reaction (20 µl) was carried out in a solution of 150 ng of ^32P-labeled dsDNA or ^32P-end-labeled dsDNA, 2 mM ATP, and 1.5 µg (3.1 µM) of RecR protein in buffer F (50 mM HEPES-KOH, pH 7.0, 100 mM KCl, 2 mM MgCl₂, 1 mM ZnSO₄, 0.1 mM EDTA, 0.1 mM DTT) and incubated for 5 min at 20 °C. The binding reactions were performed in buffer F, unless stated otherwise.

The chemical treatment of the DNA was performed by incubating the DNA in buffer F with a given DNA damaging agent. After 5 min, ATP (2 mM) and the RecR protein (0.3 µM) were added and incubation continued for another 5 min. Ice-cold buffer F (1 ml) was added to the reaction mixture to stop it. The reaction was then filtered through KOH-treated filters. Filters were dried and the amount of radioactivity bound to the filter was determined by scintillation counting. The DNA retained on filter was digested with enzyme (e.g. S1 nuclease), and the DNA in the absence of RecR protein. The specificity of the labeled DNA was measured as trichloroacetic acid-precipitable material. All reactions were performed in duplicate.

We have found that under certain experimental conditions (low salt, high magnesium, absence of ATP, etc.) a gross aggregation of free RecR protein takes place, interfering with the analysis of the binding constants and cooperativity.

**Electron Microscopy**—The formation of RecR-DNA complexes for electron microscopy were performed either in buffer G (25 mM triethanolamine (TEA)-CIH, pH 7.5, 100 mM KCl, 0.1 mM EDTA) or buffer H (25 mM TEA-CIH, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM ZnSO₄, 0.1 mM EDTA, 0.1 mM DTT) with pUC18 DNA. The DNA was labeled with ^32P]ATP (2 mM) and 500 ng of RecR protein, in a volume of 50 µl, were incubated, either in buffer G or H, for 15 min at 37 °C. The complexes were fixed with glutaraldehyde at 0.2% final concentration for 15 min and separated from unbound RecR or glutaraldehyde by gel filtration. The specimens, present in the void volume of a Sepharose 4B-CL minicolumn (1.5 ml), were equilibrated with 25 mM TEA-CIH, pH 7.5, 5 mM MgCl₂, and then absorbed to mica as described previously (29).

For the analysis of base mismatches heteroduplex molecules were formed and spreaded on a water surface as previously described (30).

**Other Methods**—The pl of the RecR protein was determined using a sieve focusing kit as recommended by the supplier (Servalyt PreNets, Serva). The NH₂-terminal amino acid sequence was determined by Volker Kruit (Max-Planck-Institut für molekulare Genetik, Abteilung Wittmann, Berlin, Germany) with an automated Edman degradation in a pulsed-liquid phase sequenator (model 477A, Applied Biosystems). The concentration of DNA was determined using molar extinction coefficients of 8780 and 6500 M^-1 cm^-1 at 260 nm for ssDNA and dsDNA, respectively. Protein concentrations were determined by the method of Bradford (31) using bovine γ-globulin as a standard.

**RESULTS**

**Purification of RecR Protein**—The pBT56-encoded RecR protein (17) was specifically labeled with ^3H]methionine with the help of an in vivo expression system (23). The RecR polypeptide, under the expression conditions described under "Experimental Procedures," accounts for about 12% of total protein mass. In the absence of a biochemical assay for the RecR polypeptide, its purification was monitored by following radioactively labeled RecR protein (25 kDa). The expression induced 25-kDa polypeptide (apparent molecular weight = 24,298) was insoluble (Fig. 1A, lane 2). The RecR aggregates could, however, be dissolved in the presence of 2 M urea. This property was exploited in our purification scheme to release unwanted proteins. Fig. 1 shows the progressive purification of the radiolabeled RecR polypeptide. After the last purification step the (25-kDa) RecR polypeptide is more than 99% pure, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 1B, lane 7).

The amino terminus of the purified protein was sequenced by automatic Edman degradation. The sequence of the first 30 amino-terminal residues of the purified protein are in full agreement with the amino acid sequence deduced from the nucleotide sequence of the recR gene (termed previously recM gene, 17) (22).
FIG. 1. SDS-polyacrylamide gel electrophoresis of RecR protein purification. A, autoradiogram of [35S]methionine-labeled RecR protein. B, Coomassie Blue-stained 15% SDS-polyacrylamide gel (40). Lane 1, cell lysate (fraction 1); lanes 2 and 3, pellet (fraction 2) and supernatant, respectively; lane 4, pellet of fraction 2 (fraction 3); lanes 5 and 6, pellet and supernatant (fraction 4) of 2 M urea, respectively; lane 7, heparin-Sepharose 350 mM KCl elution (fraction 5). LMW, molecular mass standards (in kDa).

TABLE I

| Experimental condition | DNA retained on filter (%) | dsDNA | ssDNA |
|------------------------|----------------------------|-------|-------|
| Complete               | 34.1 (21.2)                | 78.1  |       |
| RecR                   | 2.2                       | 3.2   |       |
| ATP                    | 19.9                      | 43.6  |       |
| Zn2+                   | 20.4                      | 51.2  |       |
| Mg2+                   | 16.2                      | 48.0  |       |

The binding reactions were performed in a 20-μl volume with 0.15 μg of [3H]M13mp18 ssDNA or [32P]pUC18 linear dsDNA, 1.5 μg of RecR protein (3.1 μM) and 2 mM ATP in a standard buffer condition (buffer F: 25 mM HEPES-KOH, pH 7.0, 100 mM KCl, 2 mM MgCl2, 1 mM ZnSO4, 0.1 mM EDTA, 0.1 mM DTT), with the omission indicated at room temperature for 5 min. The DNA retained on filter was corrected for the retention of labeled DNA in the absence of RecR protein. The specific activities of [3H]ssDNA and [32P]pUC18 dsDNA were 120,000 and 200,000 cpm/μg, respectively. The quantitation of binding products were as described in Fig. 2. The rate of retained heat-denatured [32P]pUC18 linear DNA is shown in parentheses.

The isoelectric point of the RecR protein determined experimentally is 5.4 (see “Experimental Procedures”).

Characterization of RecR Activities—The ability of RecR protein to act as dsDNA or ssDNA nuclease (exo- or endonuclease), DNA helicase, and to bind to dsDNA or ssDNA was assayed. Binding to ssDNA and dsDNA were the only activities observed.

RecR is able to bind linear or circular pUC18 dsDNA to nitrocellulose membrane filters, with similar efficiency (Table I, data not shown). The formation of RecR-DNA complexes were detected when more than 10 RecR molecules per DNA particle were present in the reaction mixture (see below). The retention of either circular or linear [32P]pUC18 DNA-RecR complexes are enhanced when one of the following metal ions: Mg2+ (MgCl2), Zn2+ (ZnSO4) (Table 1; lanes 2 and 3, pellet of fraction 2) and supernatant, respectively; lane 4, pellet of fraction 2 (fraction 3); lanes 5 and 6, pellet and supernatant (fraction 4) of 2 M urea, respectively; lane 7, heparin-Sepharose 350 mM KCl elution (fraction 5). LMW, molecular mass standards (in kDa).

The RecR protein is also able to form protein-DNA complex with ssDNA, e.g. circular or HaeIII-digested, 3H-labeled M13 mp18 phage DNA or heat-denatured, linear 32P-labeled pUC18 DNA. As shown in Table I, the affinity of RecR for ssDNA is 3-4-fold lower than to dsDNA. A RecR-DNA complex formation was not detected when different unspecific 32P-end-labeled oligonucleotides, ranging in size from 17-mer to 56-mer, were used.

From those results can be inferred that: (i) the RecR protein binds preferentially to circular or linear dsDNA, (ii) maximal binding required ATP and divalent cations, and (iii) divalent cations may play a decisive role in the structure of the RecR protein.

Physical Parameters of RecR-DNA Complex Formation—The properties of DNA binding by RecR were characterized. Fig. 24 shows the effect of different KCl concentrations on the binding reaction. The rate of RecR-DNA complex formation increased 1.5-fold as the KCl concentration was raised from 35 to 100 mM. At KCl concentrations above 150 mM, the apparent binding constant decreased linearly.

Titration with MgCl2 or ZnSO4 showed a peak at 2 mM (Fig. 2B) and 1 mM (Fig. 2C), respectively. The rate of RecR-DNA complex formation seems to be less sensitive to higher concentrations of ZnSO4 than to MgCl2 (see Fig. 2, panels B and C).

As revealed in Fig. 2D, the RecR-DNA complex formation is increased about 2-fold when ATP or ADP, at a final concentration of 2 mM, were present in the reaction buffer; dATP has the same effect in the binding reaction (data not shown). ATPγS from 0.2 to 2 mM concentration (Fig. 2D) exerted a negative effect in the RecR-DNA complex formation, and such an effect is released at higher ATPγS concentration, presumably due to contaminating nucleotides (see “Experimental Procedures”).

The binding of RecR to DNA in the standard reaction buffer (Buffer F plus 2 mM ATP) is complete after 2–3 min (Fig. 2E) and is relatively insensitive to variations in pH, within the range from pH 4 to pH 8 (Fig. 2F). Under these conditions the binding of RecR to DNA has a broad optimal temperature of incubation, between 20 and 65 °C (Fig. 2G). Furthermore, heat treatment of the protein alone showed that DNA-binding activity was drastically decreased after 5 min of incubation at temperatures above 65 °C (data not shown).

The RecR-DNA complex was formed in buffer F plus addition of 2 mM ATP for 5 min, and then the RecR-DNA complexes were challenged, during 5 min, with increasing concentrations of unlabeled M13 phage DNA (ssDNA), supercoiled or linear pUC18 DNA (dsDNA). As shown in Fig. 2H, the RecR-DNA complex is stable in the presence of a 60-fold excess of unlabeled DNA.

The dissociation rate of the RecR-DNA complex was also measured. Following its formation in the standard binding buffer for 5 min, it was challenged with a 50-fold excess of unlabeled DNA substrate for different periods of time. Very little dissociation of the initial complex was observed during a 2-h incubation in the presence of 50-fold excess of unlabeled DNA at 20 °C. In the absence of divalent cations and ATP, however, those initial complexes are readily competed by cold DNA (data not shown).

From these results we designed our reaction buffer (buffer F: 25 mM Hepes-KOH, pH 7.0, 100 mM KCl, 2 mM MgCl2, 1 mM ZnSO4, 0.1 mM EDTA, 0.1 mM DTT) plus the addition of 2 mM ATP. All reactions were carried out in a 20-μl volume and incubated for 5 min at 20 °C, unless stated otherwise.

DNA-binding Activity of the RecR Protein—RecR is able to bind linear pT181 (G + C content 32%) or pUC18 (G + C content 50%) dsDNA to nitrocellulose membrane filters with similar affinity (Fig. 3). The RecR-DNA complex retained by the filter is less than unity. In the plateau region where all the DNA is presumably saturated with RecR, 75–85% retention is observed. The same results are obtained when M13 mp18 replicative form DNA is used. Since an identical region longer than 9 nucleotides was not detected between the published sequences of both DNA substrates used, we could assume that such a binding is not nucleotide sequence specific (see below).

The rate of RecR-DNA complex formation was determined
The physical parameters of RecR binding to DNA. The RecR binding parameter measured is described inside each panel. Analytical binding reactions were performed in a 20-μl volume with 0.15 μg of linear [32P]pUC18 dsDNA and 1.5 μg of RecR protein in binding buffer during 5 min. This incubation mixture was then diluted with 500 μl buffer F and filtered in a filter holder. Except as otherwise indicated in each panel, buffer F plus addition of ATP (2 mM) was used. The specific activity of pUC18 DNA was 220,000 cpm/μg. The DNA retained on filter was corrected for the retention of [32P]pUC18 DNA in the absence of RecR protein (2-4% of total input).

As a function of RecR protein concentration (Fig. 3). At a protein concentration of about 0.1–0.25 μM, no binding was detected, whereas at higher RecR concentrations the dependence of complex formation is sigmoidal. The apparent equilibrium constant, which in this case is equal to protein concentration midpoint, is 9 × 10^-7 M. The results suggest that RecR protein binds dsDNA in a cooperative manner.

Visualization of the RecR-DNA Protein Complexes—Electron microscopy was used to investigate the interaction of purified B. subtilis RecR protein with supercoiled pUC18 DNA. Experiments were designed to examine the RecR-DNA complexes in the presence (buffer H) or absence (buffer G) of divalent cations. Under these experimental conditions, the requirement for nucleotide cofactors was also assayed.

As shown in Fig. 4A, incubation of the RecR protein with circular duplex DNA in buffer G, yields discrete complexes of RecR bound to several positions in a single DNA molecule. Such an effect cannot be attributed to saturation of the substrate, because free DNA is still detected. The RecR-DNA complex formation, under these experimental conditions, is independent of the presence of nucleotide cofactors (Fig. 4, A–D). The RecR co-aggregates seem to be globular in shape and uniform in size.

At high protein concentrations a nearly uniform covering of the duplex DNA was observed (data not shown). The RecR does not form filaments or rod structures even at a protein concentration (5 μM) 10 times higher than the one presented in Fig. 4.

Incubation of RecR with DNA in buffer H, leads to the formation of different types of complexes, and those are influenced by the addition of nucleotide cofactors. The presence of 2 mM ATP (Fig. 4F) or dATP favored the formation of one protein complex per DNA molecule. The RecR co-
aggregate average width is, however, significantly larger than in the absence of divalent cations (see above). Addition of 2 mM ADP favored the formation of large RecR co-aggregates that could trap more than one DNA molecule (Fig. 4G).

As revealed in Fig. 4E, the presence of divalent cations favored the formation of large assemblies of RecR, which still retains its DNA binding activity. Addition of 2 mM ATPγS, to the above conditions seems to inhibit the DNA binding activity of the RecR protein (Figs. 2 and 4H). The RecR clumps formed in the presence of ATPγS show only occasionally adhering DNA molecules.

On the basis of the results presented in this and previous sections we assumed that in the absence of divalent cations the binding of RecR to DNA is unspecific, and it was not further analyzed.

The incubation of RecR protein with either the replicative form of M13 mp18 or linear pUC18 DNA yielded results identical to those presented above. The RecR-DNA complexes, when formed in buffer H and in the presence of ATP, present one protein aggregate per DNA molecule (Fig. 4F). A physical mapping of these complexes fails to show any specific binding region in the DNA molecule. Furthermore, the length of the DNA does not seem to be modified by the presence of the RecR protein (data not shown).

When RecR was incubated with mixed ssDNA (phage M13 mp18 DNA) and dsDNA (M13 mp18 replicative form), dsDNA-RecR complexes are readily formed. The RecR protein shows a 3–4-fold higher affinity for dsDNA than for ssDNA (see above). The RecR co-aggregates on ssDNA molecules are also globular in shape and uniform in size (data not shown). The binding of RecR to ssDNA was not further analyzed in this report.

RecR is a DNA-binding Protein with Affinity for Damaged DNA—The results presented in the previous sections suggest that the B. subtilis RecR protein is able to recognize a structure or unusual deformability on the DNA rather than a specific nucleotide sequence. To test this hypothesis the DNA substrate was preincubated (5 min) with a given chemical mutagen, and then the amount of RecR-DNA complex formation was measured. The lesions generated by the different chemical mutagens used include the generation of purine adducts (4-nitroquinoline-1-oxide), (O'-methyl(ethyl)guanine alkylpyrimidines (methyl methanesulfonate and ethyl methanesulfonate), N³-hydroxy (A,C,U) substitutions on stretches of ssDNA (hydroxylamine hydrochloride), induction of cross links (mitomycin C) or intercalation into the DNA (ethidium bromide) (see Ref. 32).

When the RecR protein is present in limiting amounts (0.9 μM) the rate of RecR-DNA complex formation can be enhanced by pre-damaging the DNA substrate with different chemical agents (Table II). Since the chemical agents generate different products we could infer that the RecR protein does not recognize damaged bases per se but rather recognizes generalized damage to DNA. The common feature in such damaged DNA could be a distortion of the helix axis of the DNA (local deformability). To determine the relative affinity of the RecR protein for damaged duplex DNA substrates, linear damaged or undamaged dsDNA were used. The binding reactions were performed in the presence of various DNA concentrations (either damaged or undamaged), while the RecR protein amount was kept constant (0.9 μM). As shown in Fig. 5, the relative amount of the DNA-RecR retained on the filter remained constant at low DNA concentrations. At a concentration of 4.2 nM about half of the undamaged duplex DNA substrate is not retained by RecR in the nitrocellulose filter. In the case of damaged DNA a concentration of 12.5 nM is needed to reach the same amount of free DNA. The protein concentration midpoint of RecR for damaged DNA is of the order of 3 × 10⁻⁷ M and 9 × 10⁻⁷ M for undamaged DNA.

Electron microscopy was also used to study the interaction of the RecR protein with either damaged or base mismatched DNA. The shape and size of the RecR co-aggregates on DNA which was preincubated with 4-nitroquinoline-1-oxide (250 μM), are indistinguishable from those presented in Fig. 4. Here also ATPγS exerted a negative effect on RecR binding to DNA (data not shown). Base mismatches cause helical deformations. We analyzed the RecR binding to DNA heteroduplexes containing one mismatch (A/C), base pair insertion or two base pair insertion (DNA loops). In both cases we failed to detect any specific binding that can be ascribed to the mismatch (data not shown).

### Table II

| Substrate specificity of RecR-DNA binding activity | Experimental condition* | DNA retained on filter |
|--------------------------------------------------|-------------------------|-----------------------|
|                                                   |                         | %                     |
| -Damage                                          |                         | 39.0                  |
| +4-Nitroquinoline-1-oxide                        |                         | 81.5                  |
| +Methyl methanesulfonate                         |                         | 74.5                  |
| +Ethyl methanesulfonate                          |                         | 82.7                  |
| +Hydroxylamine hydrochloride                     |                         | 79.9                  |
| +Mytomycin C                                     |                         | 59.9                  |
| +Ethidium bromide                                |                         | 44.8                  |

*The binding reactions were performed in a 20-μl volume with 150 ng of [³²P]pUC18 linear dsDNA and 435 ng of RecR protein in buffer F plus the addition of 2 mM ATP. The DNA was preincubated with the indicated chemicals for 5 min at 20 °C. The DNA retained on the filter was corrected for the retention of [³²P]DNA in the absence of RecR protein. The specific activities of [³²P]DNA was 200,000 cpm/μg. The quantitation of binding products were as described in Fig. 2. The 4-nitroquinoline-1-oxide (250 μM), methyl methanesulfonate (100 μM), ethyl methanesulfonate (250 μM), hydroxylamine hydrochloride (50 μM), mytomycin C (250 μM), and ethidium bromide (0.15 μg/μl) were added 5 min prior to the addition of 2 mM ATP and RecR. The presence of the damaging agents does not affect the binding reaction (data not shown).

**FIG. 5.** Binding of RecR protein to damaged and undamaged pUC18 dsDNA. Analytical binding reactions were performed in the presence of a constant amount of RecR protein (435 ng) in buffer F plus addition of 2 mM ATP. Filled circles denote the undamaged DNA, and open circles the pUC18 DNA, which was preincubated with 250 μM 4-nitroquinoline-1-oxide. The DNA retained on filter was corrected for the retention of [³²P]DNA in the absence of RecR protein (2–5% of total input). The quantitation of binding products was as described in Fig. 2.

**DISCUSSION**

Genetic evidence suggests that the RecR protein from both *B. subtilis* and *E. coli* is an essential part of the α or RecF pathway (1–3). In both bacteria, the recR gene encodes a polypeptide that migrates on SDS gels as a 25 kDa protein.
Two putative DNA binding (helix-turn-helix and zinc finger) and a nucleotide binding motif have been deduced from the protein primary structure (22, 33). The recR gene product from *B. subtilis* 168 has been purified. The purified RecR protein (25 kDa, pl 5.4) is able to bind ssDNA and dsDNA. The data show that in the presence of ATP and divalent cations, the apparent equilibrium constant for undamaged DNA is $9 \times 10^{-7}$ M and for damaged DNA is of the order of $3 \times 10^{-7}$ M. The RecR protein binds dsDNA in a cooperative manner. Damage recognition by RecR, however, is an extremely low selectivity process, hence we have to assume that other component(s) might increase the selectivity in the formation of the nucleoprotein complex.

Two distinct features of the RecR protein have been observed. In the absence of divalent cations, RecR protein forms multiple aggregates in a single DNA molecule. The formation of such complexes is independent of nucleotide cofactors and readily competed by cold DNA. We could speculate that in this condition, RecR binding to DNA is stabilized by non-specific contacts via its putative helix-turn-helix motif (see 33). In the presence of divalent cations, the addition of ATP or dATP favored the formation of one RecR self-assembly per DNA molecule. Under these experimental conditions RecR shows its higher affinity for DNA and the rate of protein-protein interaction is markedly enhanced. This is consistent with the prediction that a putative domain for nucleotide binding is present in the RecR protein (22).

Which is the role of a nucleotide cofactor in RecR binding to DNA? Since ATP, dATP and ADP enhanced the rate of RecR binding to DNA we could hypothesize that those nucleotides could have an "allosteric effect" on RecR specific binding to DNA, and it binds to damaged DNA (specific binding) via the putative zinc finger motif (22). This hypothesis is consistent with the following observations: (i) RecR specific binding is achieved through nucleotide binding rather than its hydrolysis, and (ii) the substitution of one of the cysteine residues by a serine in the potential finger site resulted in a decrease in the survival of the mutant strain.²

² A. C. Stiege and J. C. Alonso, unpublished results.

The binding of the RecR protein to dsDNA described here, parallels in several aspects the DNA binding ability of other proteins involved in the specific binding to damaged DNA as the *E. coli* UvrA protein (see 34) and the mammalian excision repair XPc protein involved in xeroderma pigmentosum (35, 36). Except for UvrA which contains two, these proteins contain a single sequence of the form Cx3Cx6CxC ("Zn finger motif", reviewed in Ref. 37). The common feature in the damage repertoire recognized by these proteins could be a distortion of the helix axis (local deformability). The proteins require detection of an attribute that distinguishes damaged from undamaged DNA and independent of the intrinsic structure of the lesion. The proteins could recognize structures as DNA kinking, as was found in the case of the UvABC protein for cisplatin adducts (38). Both the *E. coli* UvrABC protein and the *B. subtilis* RecR protein, however, do not act on base mismatches (Ref. 16 and this report).

Homologous recombination by the RecBCD pathway is initiated at a DNA sequence known as Chi present on a linear duplex DNA (9). Conversely, recombination by the RecF or α pathway could be initiated by the presence of local deformation on the DNA. The RecR, as the UvrA protein, has affinity for damaged dsDNA and apparently is devoid of DNA helicase activity. We could hypothesize that RecR delivers the RecF and RecL(RecO) proteins, which are thought to function together with RecR (see Introduction), onto the damaged site. This heteroprotein complex might have a DNA-dependent nuclease and helicase activity associated with it. Upon recognition of the damaged site heterosubunit protein could generate a DNA intermediate that could work as the major SOS inducing signal. This is consistent with the fact that mutations affecting the recf, recO(RecL) and recR gene product markedly reduced SOS induction (11, 14, 17, 39).

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