Spore Photoprodut (SP) Lyase from *Bacillus subtilis* Specifically Binds to and Cleave SP (5-Thyminyl-5,6-Dihydrothymine) but Not Cyclobutane Pyrimidine Dimers in UV-Irradiated DNA

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Endospores of *Bacillus subtilis*, as well as other *Bacillus* and *Clostridium* spp., are significantly more resistant to 254-nm-wavelength UV radiation than are their exponentially growing counterparts. Spore UV resistance is due to the unique UV photochemistry of spore DNA and the efficient repair of spore DNA damage during germination (reviewed in references 14, 15, and 23). In the spore or in vitro, binding of spore DNA by small, acid-soluble spore proteins (SASP) of the *B. subtilis* class results in an alteration of the DNA helical conformation of dormant-spore DNA from the B form to an A-like form (10). UV irradiation of either spores (1, 22) or SASP-DNA complexes in vitro (16) favors production in DNA of the unique spore photoprodut (SP) 5-thyminyl-5,6-dihydrothymine and suppression of cyclobutyl pyrimidine dimer (Py–Py) formation (references 1 and 6; reviewed in references 23 and 24).

An important determinant of SP repair during spore germination is its direct reversal to two thymines in DNA by the enzyme SP lyase, encoded by the *splB* gene in *B. subtilis*. SplB protein containing an N-terminal tag of six histidine residues [(6His)SplB] was purified from dormant *B. subtilis* spores and shown to efficiently cleave SP but not cyclobutane cis,syn thymine-thymine dimers in vitro. In contrast, SplB protein containing an N-terminal 10-histidine tag [(10His)SplB] purified from an *Escherichia coli* overexpression system was incompetent to cleave SP unless the 10-His tag was first removed by proteolysis at an engineered factor Xa site. To assay the parameters of binding of SplB protein to UV-damaged DNA, a 35-bp double-stranded oligonucleotide was constructed which carried a single pair of adjacent thymines on one strand. Irradiation of the oligonucleotide in aqueous solution or at 10% relative humidity resulted in formation of cyclobutane pyrimidine dimers (Py–Py) or SP, respectively. (10His)SplB was assayed for oligonucleotide binding using a DNase I protection assay. In the presence of (10His)SplB, the SP-containing oligonucleotide was selectively protected from DNase I digestion (half-life, >60 min), while the Py–Py-containing oligonucleotide and the unirradiated oligonucleotide were rapidly digested by DNase I (half-lives, 6 and 9 min, respectively). DNase I footprinting of (10His)SplB bound to the artificial substrate was carried out utilizing the 32P end-labeled 35-bp oligonucleotide containing SP. DNase I footprinting showed that SplB protected at least a 9-bp region surrounding SP from digestion with DNase I with the exception of two DNase I-hypersensitive sites within the protected region. (10His)SplB also caused significant enhancement of DNase I digestion of the SP-containing oligonucleotide for at least a full helical turn 3′ to the protected region. The data suggest that binding of SP lyase to SP causes significant bending or distortion of the DNA helix in the vicinity of the lesion.

SPI lyase during spore germination proceeds in the absence of photoreactivating light (11, 12). The first clue to the enzymatic mechanism of SP lyase came from examination of the deduced amino acid sequence of the *B. subtilis* SplB protein. The 342-amino-acid sequence of SplB was observed to contain only four cysteines, three of which were tightly clustered at residues 91, 95, and 98 (2). The SplB sequence surrounding residues C91, C95, and C98 was found through sequence database searching to be highly similar to the amino acid signature for the [4Fe-4S] clusters of a family of *S*-adenosylmethionine (SAM)-dependent, radical-utilizing enzymes represented by anaerobic (type III) ribonucleotide reductase, pyruvate-formate lyase, lysine-2,3-ammonomutase, biotin synthase (BioB), and lipoic acid synthetase (LipA) (13, 15, 20). SP lyase activity was purified from *B. subtilis* spores expressing an engineered *splB* gene encoding a tag of six histidine residues at its amino terminus [(6His)SplB] (20). (6His)SplB was able to cleave SP in vitro, and its activity was dependent upon reducing conditions and SAM, but the protein was present in spores in exceedingly small quantities (20). Another version of SplB containing a more complex N-terminal tag consisting of 10 histidines and a factor Xa cleavage site, called here (10His)SplB, was engineered, overproduced, and purified in large amounts from *Escherichia coli* (20). The purified (10His)SplB protein was shown to contain an intact FeS cluster but was incapable of cleaving SP in vitro (20); furthermore, until recently, the 10-His tag was refractory to proteolytic cleavage with factor Xa. In this communication, we report that successful cleavage of (10His)SplB by factor Xa restores the
enzyme to an active conformation; thus, active SP lyase consists of only SplB protein. Furthermore, SP binding and SP cleavage activities can be separated by the presence of the 10-His tag on SplB.

In order to better understand at the molecular level how SP lyase binds to SP, we report here the construction of a synthetic 35-bp double-stranded oligonucleotide which contains a single pair of adjacent thymines which can be manipulated to form either T<sup>C</sup>-T or T<sup>C</sup>-T. We report that (10His)SplB protein purified from an E. coli overexpression system (i) binds specifically to the oligonucleotide containing T<sup>C</sup>-T (ii) protects SP from digestion with DNase I, and (iii) dramatically alters the DNase I footprint of the SP-containing oligonucleotide.

### MATERIALS AND METHODS

**Sources of SplB protein and SP lyase assay.** (6His)SplB was purified from dormant spores of B. subtilis strain WN417 (metC14 sul dplA48)ermC splA::(his6)iplR::cmlA E. coli thyA1 thyB1 tcpC2 as described in detail previously (20). Due to the exceedingly small quantity of the protein present in dormant spores, purification of (6His)SplB was monitored by Western blot analysis as described in detail previously (20). The N-terminal amino acid sequence of (6His)SplB was determined to be MHHHHHHNQVY by nucleotide sequencing of the cloned engineered splB gene (the italicized residues denote the natural SplB sequence [2])

**10His SplB** was overexpressed and purified from E. coli strain AD494[DE3] carrying plasmid pCLK201 essentially as described previously (20), with the modifications described below in the case of its preparation for factor Xa cleavage. The purified protein was judged to be >99% pure on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (data not shown). The N-terminal sequence of (10His)SplB was determined by Edman degradation to be MGHHHHHHHHHHSSGHIEGR/H after 15 min, DNase I (1 U) (Promega, Madison, Wis.) was added to the mixture and incubation was continued at 37°C. At 0, 5, 10, 20, 30, and 60 min after DNase I addition, the reactions were stopped by addition of Na<sub>2</sub>EDTA to a final concentration of 25 mM. Samples were electrophoresed on a 20% PAGE, stained with ethidium bromide, destained with 1× Tris-acetate EDTA electrophoresis buffer, and visualized by UV transillumination (21). Negative digital images of the gels were scanned, and band intensities were quantitatively analyzed with NIH Image software (National Institutes of Health, Bethesda, Md.).

**DNase I footprinting experiments.** The 35-base single-stranded oligonucleotide containing two adjacent thymines was 5’-end-labeled with 125 Ci of [γ-32P]ATP and T4 polynucleotide kinase (Promega). The labeled strand was then hybridized to its complementary strand, air dried, and equilibrated for 2 to 4 days in the presence of saturated ZnCl<sub>2</sub> as described above. The labeled oligonucleotide was irradiated at 10% RH with 254-nm-uv UV-irradiated double-stranded oligonucleotide was irradiated to produce SP and purified after electrophoresis through a 12% nondenaturing polyacrylamide gel. The resulting protein was activated and assayed for SP lyase activity as described above and (20).

**Sources of DNA for assay.** B. subtilis chromosomal DNA was labeled by growth in the presence of 5-methyl-[13H]Hymenin. Chromosomal DNA of B. subtilis strain WN175 containing either SP or Py was added to UV-irradiated spores or vegetative cells, respectively, as described previously (26). The DNA concentration was determined by fluorescence assay using PicoGreen reagent (Molecular Probes). Quantitation of SP or Py in UV-treated DNA was performed as described below.

**Construction and labeling of the 35-bp oligonucleotide.** Two complementary 35-mer oligonucleotides were synthesized (Genosys, Inc.), one of which contained a single pair of adjacent thymines (denoted in boldface type): 5’-CCCC GGATGATGTAGGTTAAGCTCAGTGGAATG-3’ and 5’-GATGTAGGTTAAGCTCAGTGGAATG CCCGGG-3’. The oligonucleotides were resuspended in sterile distilled water, quantitated by their absorbance at 260 nm, and hybridized by mixing them in equimolar proportions, heating them to 90°C, and cooling them slowly to room temperature in a water bath. Thymine residues in the 35-bp double-stranded oligonucleotide were radioabeled in a PCR amplification reaction using the PCR primers 5’-CCCCGGATGATGTAGGTTAAGCTCAGTGGAATG-3’ and 5’-GATGTAGGTTAAGCTCAGTGGAATG CCCGGG-3’. The PCR products were quantitated by scintillation counting and by fluorescence assay for DNA (PicoGreen; Molecular Probes) in a Turner Model 20 fluorescence spectrophotometer.

**UV irradiation conditions.** All UV treatments were performed using a short-wave UV lamp (Model UVS-11; UV Products) which emits mainly monochromatic 254-nm-wavelength UV light. The lamp output was determined using a UV/VIS spectrophotometer (Model Cary 2E; Bio-Rad). All oligonucleotides were irradiated at a final dose of 16 kJ/m². The double-stranded 35-bp oligonucleotide was irradiated in water to produce cyclobutane pyrimidine dimers (Py<sup>C</sup>-Py). To produce SP, the double-stranded oligonucleotide was first air dried from water onto a single layer of Saran Wrap, which transmits approximately 80% of incident 254-nm-wavelength radiation (29). The sample was inverted over a saturated solution of ZnCl<sub>2</sub>, sealed, allowed to air dry at room temperature for over a period of 4 to 7 days (19), and irradiated through the Saran Wrap and resuspended from the dried state into water.

**Identification of DNA photo products.** Photo-products were identified essentially as follows (26). UV-irradiated double-stranded oligonucleotides (10<sup>-24</sup> cm<sup>3</sup>/mol) were purified by HPLC to a high degree of purity (>95%). The DNA was denatured by heating to 80°C for 10 min, and then cooled to 25°C. The DNA sample was centrifuged at 1500 g for 10 min, and the supernatant was dried down to a final volume of 10 µl. The DNA was resuspended in 50 µl of 100 mM Tris-HCl (pH 8.0), 20 mM MgCl<sub>2</sub>, and 20 mM dithiothreitol, resolved in a 1:1 molar ratio of pGEM-3Zf to the DNA. After centrifugation, the supernatant was removed and the DNA was precipitated with 1.8 volumes of ethanol. The DNA precipitate was harvested by centrifugation, air dried, and resuspended in 5 µl of 100 mM Tris-HCl (pH 8.0), 20 mM MgCl<sub>2</sub>, 20 mM dithiothreitol, and 280 ng of 5’-end-labeled 35-bp double-stranded oligonucleotide. Freshly-prepared (10His)SplB (0.5, 2.5, and 5 µg of protein) was added and prebound to the oligonucleotide at 37°C for 15 min. DNase I digestion was stopped by adding Na<sub>2</sub>EDTA to a final concentration of 25 mM. The DNase I digestion products were precipitated by the addition of 1 µl of glycogen (20 mg/ml), 25 µl of 4 M lithium chloride, and 0.5 µl of 95% ethanol and incubation at 70°C for 1 h. The DNA precipitate was harvested by centrifugation (12,000 x g; 3 min; 4°C), air dried, and resuspended in 5 µl of DNA sequencing buffer (U.S. Biochemical, Cleveland, Ohio). The DNA was electrophoresed through 12% polyacrylamide sequencing gels in parallel in the G- and C- T-specific chemical sequencing reactions (7a) performed in parallel on the oligonucleotide. The electrophoresis products were visualized by autoradiography and scanned, and band intensities were quantitated using NIH Image software.

### RESULTS AND DISCUSSION

**Activation of SP lyase activity in (10His)SplB by proteolytic removal of its 10-His tag.** (6His)SplB protein purified from spores of B. subtilis strain WN417 efficiently monomerized SP in UV-irradiated spore DNA in a concentration-dependent manner (20) (Fig. 1). In contrast, (10His)SplB purified from the E. coli overexpression system was inactive in cleavage of SP (Fig. 1), and it was further noted that the 10-His tag was refractory to proteolytic removal from SplB. However, by performing factor Xa cleavage of (10His)SplB under anaerobic conditions, we were able to remove the 10-His tag from approximately 20% of the SplB molecules purified from the E. coli overexpression system. The resulting factor Xa-treated SplB preparation was then assayed for SP lyase activity and was shown to cleave SP, also in a concentration-dependent manner (Fig. 1). The results clearly indicated that the presence of the 10-His tag was preventing (10His)SplB from expressing SP lyase activity. Because (10His)SplB was the only B. subtilis protein overexpressed and purified from the E. coli system, the results also clearly indicated that SP lyase activity derived

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soley from the splB gene product after removal of its 10-His tag.

SP lyase discriminates between SP and Py<->Py. In order to test whether SP lyase specifically recognizes and cleaves SP, (10His)SplB protein isolated from the E. coli overexpression system and cleaved with factor Xa was incubated with a substrate consisting of B. subtilis chromosomal DNA which contained a mixture of cis.syn T<>T, cis.syn C<>T, and SP. Quantitation of chromatograms of the TFA hydrolysates resulting from the reactions showed clearly that proteolytically treated (10His)SplB protein cleaved only SP and not cis.syn T<>T or cis.syn C<>T (Table 1), indicating that SP lyase exhibited specificity for SP in vitro. This notion was confirmed in a time course experiment, where (6His)SplB isolated from dormant spores of B. subtilis strain WN417 was shown to cleave SP, but not cis.syn T<>T, in a time-dependent manner (Fig. 2).

UV photochemistry of the 35-bp double-stranded oligonucleotide. In order to study the interaction of SplB protein with SP in DNA, a synthetic 35-bp double-stranded oligonucleotide was designed and constructed as described in Materials and Methods. Thymine residues in the 35-bp double-stranded oligonucleotide were labeled with 3H at their 5-methyl positions by PCR amplification, and the UV photochemistry of the synthetic 35-bp double-stranded oligonucleotide was probed by

TABLE 1. Specificity of SP lyase for SP in vitro

<table>
<thead>
<tr>
<th>Photoprotect</th>
<th>SplB added (μg)</th>
<th>Amt of photoprotect</th>
<th>% Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>0</td>
<td>3.58 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>cis.syn C&lt;&gt;T</td>
<td>0.63</td>
<td>3.13 ± 0.13</td>
<td>12.5%</td>
</tr>
<tr>
<td>cis.syn T&lt;&gt;T</td>
<td>0.63</td>
<td>3.81 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>cis.syn T&lt;&gt;T</td>
<td>0.00</td>
<td>4.10 ± 0.21</td>
<td>NS*</td>
</tr>
<tr>
<td>cis.syn C&lt;&gt;T</td>
<td>0.00</td>
<td>0.59 ± 0.06</td>
<td>NS*</td>
</tr>
<tr>
<td>cis.syn C&lt;&gt;T</td>
<td>0.00</td>
<td>0.60 ± 0.00</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* Factor Xa-cleaved (10His)SplB protein purified from the E. coli overexpression system (3.12 μg of total protein containing 0.63 μg [20%] of active SplB) was incubated with 4.1 μg of B. subtilis chromosomal DNA containing a mixture of SP, cis.syn T<>T, and cis.syn C<>T for 60 min at 30°C. The results are averages of duplicate determinations.

** Significant decrease in SP by ANOVA (P = 0.07).

** NS, not significantly different from control by ANOVA.

assaying thymine-containing photoproducts produced after UV irradiation either in aqueous solution or at 10% RH. Quantitation of the TFA hydrolysis products after their separation by chromatography revealed that the unirradiated 35-bp double-stranded oligonucleotide contained no photoproducts while the 35-bp double-stranded oligonucleotide irradiated in aqueous solution accumulated Py<->Py in the form of cis.syn T<>T, trans.syn T<>T, and U<>T (the TFA hydrolysis breakdown product of C<>T) (1, 16) at 0.6, 0.55, and 0.3% of total thymine, respectively. In sharp contrast, UV irradiation of the 35-bp double-stranded oligonucleotide at 10% RH resulted in formation of SP to approximately 5.0% of total thymine. To confirm that the SP-containing oligonucleotide did not contain Py<->Py, it was 5' end labeled with [32P]ATP and polynucleotide kinase and treated with T4 endonuclease V (Epicentre, Madison, Wis.), which cleaves the phosphodiester backbone 5' to cyclobutane dimers (2a), and the reaction products were analyzed by autoradiography after electrophoresis through a 12% sequencing gel. No phosphodiester backbone cleavage of the oligonucleotide by T4 endonuclease V was detected (data not shown), indicating that (i) no significant quantities of Py<->Py were formed concomitant with SP by UV irradiation of the 35-bp oligonucleotide at 10% RH and (ii) T4 endonuclease V either does not recognize SP or its AP endonuclease activity does not function on SP.

(10His)SplB specifically protects SP-containing DNA from DNase I. Previous attempts to detect (10His)SplB binding to SP-containing DNA by gel retardation analysis were unsuccessful (data not shown). Therefore, in order to assay (10His)SplB protein for DNA binding activity, a DNase I protection assay was devised. Unlabeled 35-bp double-stranded oligonucleotides carrying no damage, Py<->Py, or SP were prepared as described above and used to test (10His)SplB binding affinity. Purified (10His)SplB protein was incubated with double-stranded oligonucleotide at a 1:1 molar ratio, and then the mixture was probed for protein-DNA complex formation by protection from DNase I digestion as described in Materials and Methods. When the double-stranded oligonucleotides remaining after DNase I treatment were visualized by non-denaturing 12% PAGE, it was observed that the double-stranded oligonucleotides containing no photoproducts (Fig. 3A) or Py<->Py (Fig. 3B) were rapidly degraded by DNase I but the SP-containing double-stranded oligonucleotide was protected from DNase I degradation (Fig. 3C). Quantitation of the remaining 35-bp double-stranded oligonucleotides by scanning densitometry of negative digital images obtained from

FIG. 1. Assay of SP cleavage activity on SP-containing B. subtilis chromosomal DNA from UV-irradiated spores. The proteins assayed were (6His)SplB purified from spores of B. subtilis strain WN417 (triangles), (10His)SplB overproduced in and isolated from E. coli (open circles), and (10His)SplB purified from E. coli and cleaved with factor Xa (solid circles). The data are averages ± standard deviation of duplicate determinations.

FIG. 2. Kinetics of reversal of SP (solid circles) or cis.syn T<>T (open circles) by (6His)SplB protein (2 μg) purified from spores of B. subtilis strain WN417. The initial amounts of photoproducts in each reaction mixture were 2.68% SP and 2.72% T<>T (expressed as a percentage of total thymine).
three separate experiments allowed us to determine the half-life for each double-stranded oligonucleotide in the presence of (10His)SplB and DNase I. The 35-bp double-stranded oligonucleotides containing no photoproducts or containing PyPy were degraded rapidly, exhibiting half-lives of 6.1 ± 1.15 and 9.6 ± 4.25 min, respectively, while the SP-containing double-stranded oligonucleotide was degraded much more slowly, demonstrating a half-life of 58.2 ± 19.8 min. The differences in the half-lives of all of the double-stranded oligonucleotides were a direct consequence of their interactions with (10His)SplB and were not due to intrinsic differences in their DNase I susceptibilities, because in control reactions performed in the absence of added (10His)SplB protein, the double-stranded oligonucleotides containing no damage, PyPy, or SP were all degraded rapidly, exhibiting half-lives of 6.2 ± 2.56, 8.5 ± 0.90, and 6.2 ± 1.15 min, respectively (Fig. 3). By analysis of variance (ANOVA), the half-lives of the double-stranded oligonucleotides containing either no damage or PyPy were not significantly increased in the presence of (10His)SplB, but the increased half-life of the SP-containing double-stranded oligonucleotide bound to (10His)SplB was highly significant by ANOVA (P = 0.010). Therefore, despite the fact that (10His)SplB was inactive in cleaving SP (Fig. 1), the protein bound tightly and specifically to SP-containing DNA (Fig. 3).

**Binding of (10His)SplB to the SP-containing oligonucleotide dramatically alters its DNase I footprint.** In order to explore SplB binding to SP-containing DNA at a higher level of resolution, the SP-containing 35-bp double-stranded oligonucleotide was 5'-end-labeled with 32P on the SP-containing strand and complexed with (10His)SplB, the complexes were subjected to limited DNase I digestion, and the resulting DNase I footprints were analyzed. A typical autoradiogram (Fig. 4A) showed that addition of (10His)SplB dramatically altered the DNase I cleavage pattern of the SP-containing oligonucleotide and that the alteration occurred in a manner dependent upon the amount of (10His)SplB added. To quantitate the effect of (10His)SplB binding on the DNase I footprint, a digital image of the autoradiogram was subjected to densitometry (Fig. 4B), and the intensity of each band was quantitated using the program NIH Image (Fig. 4C). Analysis of the data indicated that (10His)SplB protected a region of at least 9 nucleotides (nt) extending from T14 to A22 on the SP-containing strand of the oligonucleotide from DNase I digestion (it was not possible to assess protection of DNA 5' to this point due to limitations in the resolution of the sequencing gel). In sharp contrast, DNase I digestion was dramatically enhanced on the 3' side of the protected region for at least a full helical turn from C23 to T33 (Fig. 4C), indicating that binding of (10His)SplB to the oligonucleotide induced bending, unwinding, or distortion in DNA adjacent to the (10His)SplB binding site, reminiscent of that observed in the DNase I footprints of E. coli DNA photolyase on double- and single-stranded DNA containing TPyPy (5) and in the nontranscribed strand of DNA entering vaccinia virus RNA polymerase (4). Interestingly, within the 9-nt protected region extending from T14 to A22, binding of (10His)SplB led to an enhancement of DNase I cleavage at two positions: G19 immediately 5' to SP and T20, the 3' T within SP itself. DNase I-hypersensitive sites have been detected within the DNase I-protected regions in a number of systems and are generally also attributed to bending or distortion in the DNA helix as a result of protein binding (3–6, 17, 30). Thus, it appears that binding of SP in DNA by (10His)SplB leads to significant distortion of the phosphodiester backbone, as manifested by alterations in the DNase I cleavage pattern on the damage-containing strand and the appearance of DNase I-hypersensitive sites within the protected region.

Because enzymes causing direct reversal of pyrimidine dimers, such as SP lyase, probably recognize their cognate DNA damage in every sequence context (5), it was not expected that SP lyase would make specific hydrogen bonds within either the major or minor groove within the DNase I-protected region. To explore this point further, dimethyl sulfate (DMS) footprinting was performed on complexes of (10His)SplB and the SP-containing oligonucleotide in parallel with the DMS reactions, giving rise to the “G ladder” used for identification of base positions within the oligonucleotide. Binding of (10His)SplB to the end-labeled SP-containing oligonucleotide resulted in no significant alteration in its DMS digestion pattern (data not shown).

In summary, the in situ reversal of SP by the novel DNA

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**FIG. 3. DNase I protection experiment.** The 35-bp double-stranded oligonucleotide carrying: no dimer (A), PyPy (B), or SP (C) was preincubated either with (solid symbols) or without (open symbols) (10His)SplB and then treated with DNase I and quantitated as described in Materials and Methods. The data are represented as averages ± standard deviations of three independent experiments. The horizontal dashed line represents 50% degradation.
repair enzyme SP lyase during germination of UV-irradiated B. subtilis spores is a major determinant of spore UV resistance (14, 15), and an essential first step in SP repair is accurate recognition of and binding to the adduct. In this communication, we showed that (10His)SplB protein purified from an E. coli overexpression system bound to a 35-bp double-stranded DNA oligonucleotide containing a single SP but was unable to complete catalysis unless its N-terminal 10-His tag was first proteolytically removed at the engineered factor Xa site (Fig. 1). Thus, the SP-specific binding and cleavage functions of SP lyase are separable. Factor Xa-cleaved (10His)SplB was able to complete catalysis and specifically cleaved SP but not cis, syn T-T. DNase I protection experiments revealed that the lack of SP lyase activity of (10His)SplB on cyclobutane dimers is most likely due to the fact the (10His)SplB does not bind to Py-Py-containing DNA with high affinity (Fig. 3).

From this communication and previous data (13, 20), the following working model for SP lyase is proposed (Fig. 5). SP lyase specifically recognizes SP in DNA. Recognition is probably sequence context independent, as binding of (10His)SplB did not alter the DMS cleavage pattern of the SP-containing 35-bp double-stranded oligonucleotide. Although the three-dimensional structure of DNA containing SP has not yet been elucidated, it is known that cis,syn T-T distorts DNA (8, 18), producing a helical kink of 27° and unwinding of 19.7° (18). Because SP lyase binds SP but not Py-Py with high affinity, it presumably recognizes an SP-specific helical distortion in DNA which differs in its geometry from the distortion caused by cis,syn T-T. Binding of SP lyase to SP apparently introduces additional distortion in the helix, as manifested by the appearance of DNase I-hypersensitive sites both within and 3' to the protected region. Enhancement in distortion of

FIG. 4. DNase I footprinting experiment. (A) Autoradiogram. Lane 1, untreated SP-containing 35-nt oligonucleotide; lanes 2 to 5, SP-containing oligonucleotide after partial DNase I digestion in the absence (lane 2) or the presence of 5.0 (lane 3), 2.5 (lane 4), or 0.5 µg (lane 5) of (10His)SplB. The molar ratios of (10His)SplB to oligonucleotide were 10:1 (lane 3), 5:1 (lane 4) and 1:1 (lane 5). The two thymines corresponding to the position of SP are shown in boldface and indicated on the autoradiogram by arrowheads. (B) Densitometric scan of lane 2 (thin line) and lane 3 (thick line) of the autoradiogram displayed in panel A. (C) Quantitative summary of DNase I footprinting experiments. The protection (upward bars) or enhancement (downward bars) of DNase I digestion at each base on the SP-containing oligonucleotide by binding of 2.5 (open bars) and 5.0 µg (solid bars) of (10His)SplB was determined relative to the unbound oligonucleotide. The positions of the two DNase I-hypersensitive sites within the footprint are denoted by asterisks. The position of SP is denoted by the two open T residues at coordinates 19 and 20. The data using 5.0 µg of (10His)SplB are the averages of two independent determinations; the error bars denote the maximum value obtained.

FIG. 5. Proposed sequence of events in SP cleavage by SP lyase. Abbreviations: Ado, adenosine; FeS, iron-sulfur cluster; met, methionine. See the text for details.
Py<>Py-containing DNA by binding of DNA repair proteins has also been observed in the Uvr(A)BC excinuclease (25), DNA photolyase (5), and phage T4 endonuclease V (7). Once SP-specific binding occurs, the [4Fe-4S] cluster of SP lyase (13, 20) interacts with SAM, presumably resulting in the creation of a 5'-adenosyl radical (28) which participates in reversal of SP to two thymines, likely by radical fragmentation (9).

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